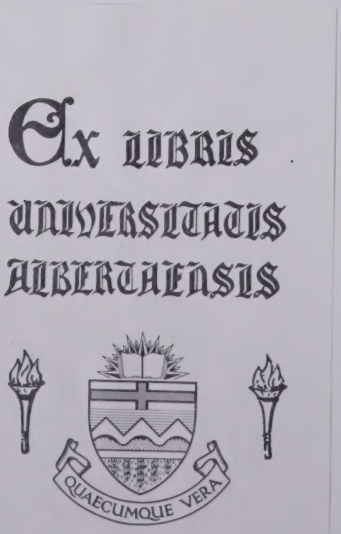

THE UNIVERSITY OF ALBERTA

VERTEBRATE SERUM INHIBITORS OF *AEDES AEGYPTI* (L.) TRYPSIN

by

CHAU-TING HUANG

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE

OF DOCTOR OF PHILOSOPHY

DEPARTMENT OF ENTOMOLOGY

EDMONTON, ALBERTA

SPRING, 1970

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies for acceptance, a thesis entitled VERTEBRATE SERUM INHIBITORS OF *AEDES AEGYPTI* (L.) TRYPSIN submitted by Chau-ting Huang in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

ABSTRACT

A trypsin from the midguts of *Aedes aegypti* (L.) has been partially purified. Its molecular weight is about 21,500. This trypsin was inhibited by the sera of 17 vertebrates and by the haemolymph of *Periplaneta americana*. The inhibition capacity is relatively high in birds and low in an elasmobranch, with mammals, reptile, frog, teleosts, and insect in the middle range. The number of inhibitors and their approximate molecular weights in each serum has been studied by Sephadex gel filtration.

Two trypsin inhibitors have been purified from bovine serum and characterized. Inhibitor I has a molecular weight of about 43,500 and inhibitor II has a molecular weight of about 1,000,000. They are located electrophoretically in association with the α_1- and the α_2-globulin fraction of serum respectively. Stoichiometric measurements indicate that the molar ratio of trypsin-inhibitor I and trypsin-inhibitor II complex is 3.5 and 1.7 respectively. The Hill plot indicates that two molecules of inhibitor (inhibitor I and inhibitor II) inactivate one enzymic site of trypsin.

In vitro, the formation of trypsin-inhibitor II complex has been demonstrated by gel filtration and cellulose acetate electrophoresis. The complex retains most of its esteratic activity but has a very low proteolytic activity. The esteratic activity of the trypsin-inhibitor II complex is not inhibited by inhibitor I, soybean trypsin inhibitor, or phenylmethanesulfonyl fluoride (PMSF). The free trypsin is inhibited by these substances. The complex has a lower K_m than the trypsin, but

both have the similar pH optimum.

Inhibitor I and II competitively inhibit the tryptic hydrolysis of denatured bovine hemoglobin. These two inhibitors and whole bovine serum non-competitively inhibit the tryptic hydrolysis of α-N-Benzoyl-DL-arginine-p-nitroanilide HCl (BAPNA) at 37 C. The mechanism of trypsin inhibition has been discussed from the aspect of the types of inhibition, and the thermodynamic parameters of the enzyme-inhibitor and the enzyme-substrate complexes.

ACKNOWLEDGEMENTS

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I am indebted to my fellow graduate students, Mr. P. K. Chiang and Mr. R. E. Leech for much advice. I thank also Miss N. Daviduk for typing the thesis.

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INTRODUCTION

In the past few years, much work has been done on the digestive physiology of blood sucking insects, since the rate of blood digestion affects the frequency of biting and possibly the ability of the insect to transmit disease. The rate of blood digestion was considered as an important factor not only in the physiology of mosquitoes but also in studies of their host relationships (West and Eligh, 1952). Many techniques have been applied to study the processes and results of blood meal digestion by mosquitoes: these include the precipitin test (Bull and King, 1923; West and Eligh, 1952; O'Gower, 1956; Williams, 1956; Downe, 1960; Zaman and Chellappan, 1967), the histological method (Huff, 1934; Bertram and Bird, 1961; Gander, 1968), the observational studies of Shlenova (1938), and of Hocking and MacInnes (1948), the crystallization method of Biocca (1950), and studies on the proteolytic activities after blood meals (Fisk and Shambaugh, 1952; Gooding, 1966 b). Many physical and biological factors, such as temperature (Shlenova, 1938; Williams, 1956), humidity (Mayne, 1928), period of light and dark during digestion (O'Gower, 1956; Gooding, 1966 b), size and source of blood meals (West and Eligh, 1952; Downe *et al.*, 1963; Langley, 1966), and certain cations and antibiotics (Terzian and Stahler, 1964) have been studied to show their effect on the rate of blood digestion in mosquitoes. It is agreed generally that the protease activity measurement combined with the chemical determination of protein content of midgut give a more accurate indication of completion of blood digestion than any other known methods.

By using specific substrates, two proteases have been demonstrated in adult female mosquitoes *Aedes aegypti* (L.) after a blood meal: a trypsin acting on Benzoyl-L-arginine ethyl ester (BAEE) and a chymotrypsin acting on Benzoyl-L-tyrosine-ethyl ester (BTEE). These two enzymes also digest the protein in the blood meal, and their general biochemical properties have been studied (Wagner *et al.*, 1961; Gooding, 1966 a).

Fisk and Shambaugh (1952) found an immediate decrease in *Aedes aegypti* protease activity below the residual value after a meal of human blood but not after a sucrose meal. They suggested the possibility of an anti-trypsin in human blood which might temporarily neutralize the mosquito trypsin more rapidly than would be done by a simple substrate depletion effect noted by Day and Powning (1949). Gooding (1966 a) found that both alkaline proteases from *Aedes aegypti* and *Culex fatigans* were inhibited by serum from normal and malarious chicks. Gooding and Huang (1969) also found that both trypsin and chymotrypsin from a ground beetle, *Pterostichus melanarius*, were inhibited by sera from cattle, sheep, pig, turkey, and chicken. These proteases were competitively inhibited by bovine serum and the inhibitor appears to be associated with the α-globulin. In fact, vertebrate sera contain inhibitors which can inhibit various vertebrate proteases specifically or in a broad spectrum (Vogel *et al.*, 1968). Inhibitors from human serum, for example, contain (1) a specific chymotrypsin inhibitor in the post-albumin region, (2) an inhibitor for trypsin and chymotrypsin in the α_1-globulin region, (3) a slow reacting plasmin inhibitor in the α_1-globulin fraction, (4) a specific trypsin inhibitor between the α_1- and α_2-globulin fractions and (5) an inhibitor for trypsin, chymotrypsin and plasmin in the α_2-globulin. From this, it becomes important to understand the nature

and behaviour of these inhibitors in the mosquito midgut during digestion of the blood meal before one can interpret the results of proteolytic activity measurements made on the mosquito midgut.

Biedermann (1898) was probably the first to note increase in digestive enzymes in larvae of *Tenebrio molitor* following feeding. In insects, three possible mechanisms of enzyme stimulation by feeding have been suggested. The first mechanism is the secretogogue mechanism in which the foodstuff itself or its products chemically stimulate secretion. This hypothesis has been considered by Fisk and Shambaugh (1952) in *Aedes aegypti* protease secretion, and it was demonstrated directly in protease secretion in the cockroach, *Leucophaea maderae* by Engelmann (1969), and in the fleshfly, *Sarcophaga bullata* by Engelmann and Wilkens (1969). The second mechanism is that the act of feeding, or the detection of food, may set up a nervous reflex to which the secretory cells respond. Fisk (1950) suggested the possibility of nervous intermediation in *Aedes aegypti*. However, this seems unlikely in view of the delayed responses noted in every case (Fisk and Shambaugh, 1952). Also, Day and Powning (1949) have shown that the midgut of *Periplaneta* and the caeca of *Periplaneta* and *Blattella* lack nerves. A third possible mechanism involves hormones which is like the nervous mechanism except that feeding results in production of a hormone which reaches the digestive tract through the haemolymph. A hormonal control of protease secretion has been demonstrated in *Calliphora erythrocephala* (Thomsen and Møller, 1963), *Tenebrio molitor* (Dadd, 1961) *Nauphoeta cinerea* (Rao and Fisk, 1965), *Locusta migratoria* (Khan, 1963), and *Glossina morsitans* (Langley, 1967). Thus it seems that the mechanisms of protease stimulation in insects are not uniform. Fisk and Shambaugh (1952)

considered that the secretogogue mechanism in *Aedes aegypti* protease stimulation was favored over the hormonal theory. They found that the protease activity was highest about 18 hours after blood feeding. Gooding (1966 b) found that the maximum protease activity occurred 24 and 36 hours after the blood meals respectively, in the midguts of *Aedes aegypti* and *Culex fatigans*. In the present study, the maximum activity of *Aedes aegypti* trypsin was found around 35 hours after a rat blood meal and 40-45 hours after a human blood meal. Therefore, in this study, the fed mosquitoes were dissected 35 to 40 hours after a rat blood meal.

STATEMENT OF THE PROBLEM

Adult females of most mosquito species require blood meals for egg production and their midgut proteases play a role in digestion of the blood meal. A study of both the mosquito proteases and the vertebrate serum inhibitors of these enzymes will add to general understanding of mosquito digestive physiology. With this in mind the specific objectives of the present study are:

1. To survey the *Aedes aegypti* trypsin inhibition capacity of several sera.
2. To study the properties of partially purified *Aedes aegypti* trypsin and inhibitors of this enzyme that are found in bovine serum.
3. To investigate some aspects of the interaction of *Aedes aegypti* trypsin with bovine serum inhibitors.

and 4. To measure protease activities and study electrophoretic patterns of blood meal proteins in the adult female mosquito midgut during the course of blood meal digestion.

MATERIALS AND METHODS

1. Materials

The mosquitoes, *Aedes aegypti* (L.), used in this study were colonized in the Department of Entomology, University of Alberta, Edmonton, Alberta, Canada. The insects were reared and maintained in the insectary at 24-27 C and 40% relative humidity. The larvae were fed commercial rabbit-food pellets, and the adults were fed 5% sucrose solution first and then on a laboratory rat. Thirty-five to 40 hours after the blood meal, the adult female mosquitoes were immobilized by chilling. The midguts were dissected out and placed in neutralized demineralized water for several hours. The solution was centrifuged at 12,100 x g for 10 minutes at 4 C, the supernatant was collected and purified as indicated in section 1 of the Experiment Section below.

Blood from cattle, pig, sheep, chick, and turkey were collected from local slaughter-houses while human blood was collected from volunteers. Blood from rat, elk, turtle, frog, and fishes were collected mainly from the Department of Zoology, University of Alberta. The blood samples were allowed to clot overnight at 4-6 C. The sera were separated after centrifugation and stored frozen until use. Haemolymph of *Periplaneta americana* was collected from cockroaches reared in the Department of Entomology, and its supernatant was kept after centrifugation. Lyophilized serum of dog, horse, and rabbit were bought from Nutritional Biochemicals Corporation, Cleveland, Ohio, U.S.A.

Bovine trypsin (B grade) was purchased from Calbiochem. Los Angeles, U.S.A. Denatured bovine hemoglobin was used as the substrate for the

mosquito proteases; α-N-Benzoyl-L-arginine ether ester (BAEE) and α-N-Benzoyl-DL-arginine-p-nitroanilide HCl (BAPNA) are the substrates for trypsin. Benzoyl-L-tyrosine ether ester (BTEE) is the substrate for chymotrypsin. These substrates were purchased from Nutritional Biochemicals Corporation, Cleveland, Ohio, U.S.A. Soybean trypsin inhibitor was bought from Worthington Biochemicals Corporation, Freehold, New Jersey, U.S.A. The trypsin inhibitors, phenylmethanesulfonyl-fluoride (PMSF) and N-α-p-Tosyl-L-lysine chloromethyl ketone HCl (TLCK), were purchased from Sigma Chemical Company, St. Louis, Missouri, U.S.A. Other chemicals and reagents were bought mainly from Fisher Scientific Co., Edmonton, Alberta, Canada; Sigma Chemical Company, St. Louis, Missouri, U.S.A.; or Pharmacia (Canada), Montreal, Canada.

2. Methods

Centrifugations were done in a Sorvall RC2-B refrigerated centrifuge at 12,100 x g for 10 minutes at 4 C. Sephadex gel filtration and ion exchange chromatography (DEAE- and CM-cellulose) were carried out at approximately 4 C in a Gilson refrigerated fraction collector. Electrophoreses were run by using cellulose acetate membrane in a Gelman Delux electrophoresis chamber at 4 C. The spectrophotometric measurements were performed in a Beckman DU-2 spectrophotometer equipped with a double thermospacer through which water was pumped from a constant temperature water bath.

All protein measurements were done according to the U.V. absorption method of Layne (1957) or the Folin reagent method of Lowry *et al.*, (1951). The molecular weights of purified enzyme and inhibitors were estimated by the

method of Andrews (1964) using the following purified proteins to calibrate the Sephadex gel columns: bovine hemoglobin (64,500), bovine serum albumin (monomer, 67,000; dimer, 134,000), β-lactoglobulin (36,000), soybean trypsin inhibitor (21,500), and α-lactalbumin (15,500).

The proteolytic activity of trypsin was measured by the hydrolysis of 10% (w/v) denatured bovine hemoglobin (in 0.9 M NaCl) at 37 C for 20 minutes. The assay procedure was modified from the method of Kunitz (1947) and Gooding (1966 a) by using 0.05 M Tris buffer (pH 7.9) and by increasing the concentration of trichloroacetic acid from 6 to 20% (w/v). The trypsin and chymotrypsin esteratic activities were estimated spectrophotometrically by the hydrolysis of 1 mM BAEE (in 0.05 M Tris buffer, pH 7.9) and 1 mM BTEE (in 5% 2-propanol, pH 7.9) respectively at 30 C (Schwert and Takenaka, 1955) in a Beckman DU-2 spectrophotometer and the absorbance changes at 256 nm were followed in a one minute assay. The mosquito trypsin and bovine trypsin esteratic activities were measured by the hydrolysis of 3 mM BAPNA in 0.05 M Tris buffer (pH 7.9) which contained no $CaCl_2$ (for mosquito assay) or 0.054 M $CaCl_2$ (for bovine assay). The reaction was run at 37 C for 20 minutes. The assay procedure is similar with Bieth *et al.*, (1968) except the enzyme activity was measured by the absorbance changes at 410 nm. In some studies, the assay was carried out at 30, 34, 44.5 C, and in 0.05 M sodium phosphate buffer (pH 7.9). All assays were run in duplicate.

The mean value, standard deviation, and simple regression analysis of the kinetics data of enzyme and inhibitors were analysed by APL/360 programs on the IBM 360/67 computer of the University of Alberta, Edmonton, Alberta, Canada (Williams, 1959; Falkoff and Iverson, 1968; Smillie, 1969; Chiang, 1970).

EXPERIMENT SECTION

1. Purification of *Aedes aegypti* trypsin

The suspension of blood fed mosquito midguts contained a large amount of undigested or partially digested blood meal proteins, and some other proteases. These proteins could affect the mosquito trypsin and the inhibition capacities of serum inhibitors *in vitro*. The purpose of purification of this enzyme is to increase its specific activity and reduce the concentration of substances which may interfere with subsequent studies.

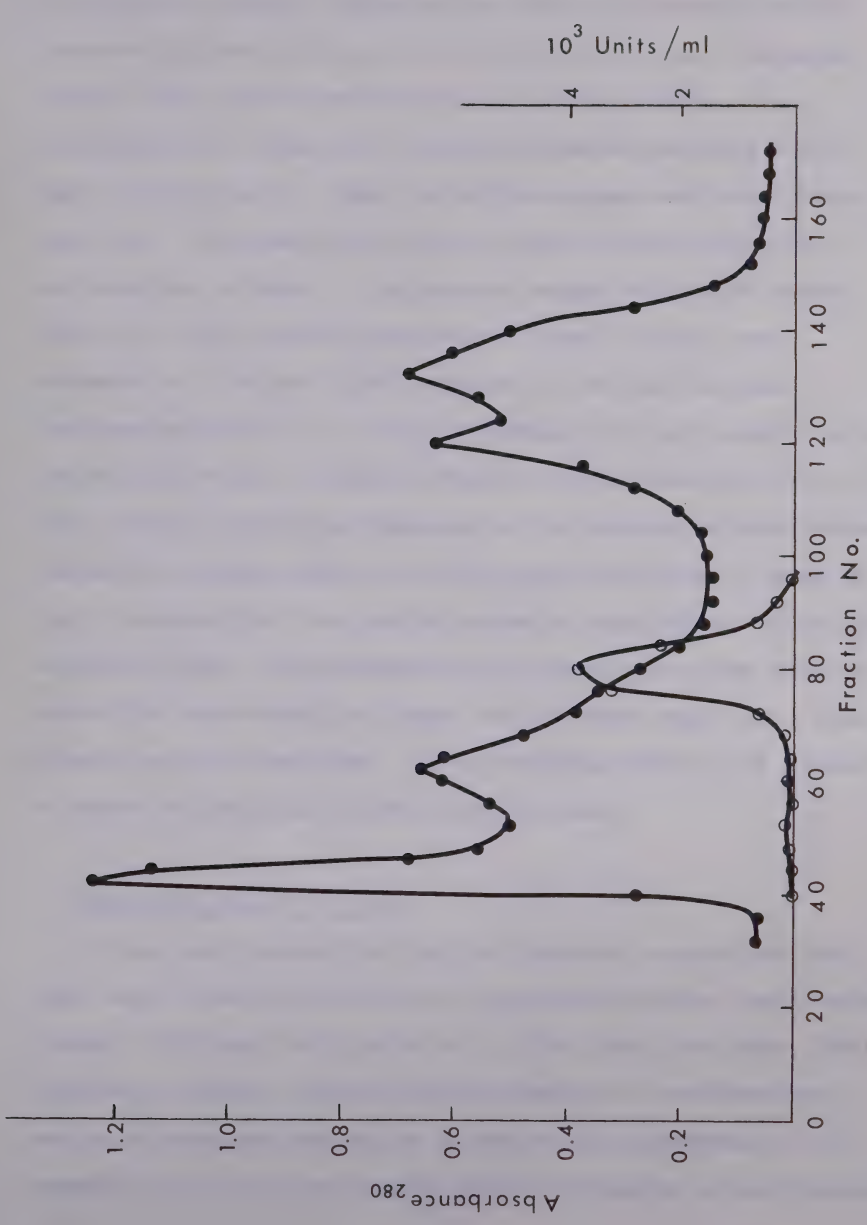
The preliminary study on the ammonium sulphate precipitation of the crude midgut suspension was carried out at 40%, 50%, 60%, 70%, 80%, 90%, and 100% (w/v) saturation, and it was found that the most tryptic activity was precipitated in the 50 to 80% SAS (saturated ammonium sulphate) fraction. Therefore in later work the fraction which precipitated between 50 to 80% SAS was collected by centrifugation and the precipitate was dissolved in 0.05 M Tris buffer containing 0.1 M KCl (pH 7.9). Each 5 ml of this solution was put onto a calibrated Sephadex G-100 gel column (1.25 x 102 cm) which was previously equilibrated with the same buffer. The flow rate was approximately 4 drops/min and the eluted solution was collected in 15 drops/fraction. The protein concentration and the BAEE tryptic activity of every fourth fraction were estimated by the methods previously described (Fig. 1). Fractions 72 to 88 (Fig. 1) were pooled and dialyzed against 0.05 M Tris buffer (pH 7.9) overnight at 4 C. The dialyzed solution was pumped onto a DEAE-cellulose column (2.5 x 18 cm) which was equilibrated with the same buffer as that

Figure 1. Sephadex G-100 chromatography of a 5 ml sample (75 mg/ml) of 0.50 to 0.80 SAS fraction of *Aedes aegypti* midgut extract.

The column (1.25 x 102 cm) was equilibrated with 0.05 M Tris-0.1 M KCl (pH 7.9) at 4 C. The flow rate was 4 drops/min and 15 drops/fraction were collected.

—●— absorbance at 280 nm

—○— trypsin activity


Absorbance 280
1.2
1.0
0.8
0.6
0.4
0.2
0
20
40
60
80
100
120
140
160
Fraction No.
10^3 Units/ml
4
2

of the enzyme solution. The enzyme was eluted by increasing the KCl concentration from 0.1 M up to 1.0 M. The flow rate was 12 drops/min and the eluted solution was collected in 54 drops/fraction. Two trypsin peaks were found after eluting with buffer containing 0.25 M and 0.5 M KCl (Fig. 2). These two purified enzymes were stored frozen until use. The enzyme specific activity and the yield of each step are summarized in Table I. The molecular weight of the first enzyme (after 0.25 M KCl) and the second enzyme (after 0.5 M KCl) was estimated as 21,500 and 17,500 respectively after they have been recolumned separately on a calibrated Sephadex G-100 gel column (Fig. 11). The molecular weight of mosquito trypsin of the crude material (Fig. 1) was 21,500 not 17,500. The comparison of the esteratic activity between the purified trypsin (after 0.25 M KCl) and bovine trypsin as shown in Fig. 3 indicates that this purified enzyme has about 80% as much activity as bovine trypsin. The difference may be because this enzyme is not as pure as the bovine trypsin or simply that the former enzyme has a lower turnover rate than the latter. In the following studies, five preparations of trypsin with molecular weight 21,500 were used.

2. Inhibition capacity of sera

It has been reported from field and laboratory observations that *Aedes aegypti* feed on several taxa of vertebrates and some insect larvae (Downes, 1958; Downe, 1960, Harris *et al.*, 1969; Harris and Cooke, 1969). The study of mosquito trypsin inhibition capacity of vertebrate sera and insect haemolymph may help us to understand the preference of this mosquito for its hosts and possibly provide information on the efficiency

Figure 2. DEAE-cellulose chromatography of active fraction obtained from Sephadex G-100 column.

The column (2.5 x 18 cm) was equilibrated with 0.05 M Tris-buffer (pH 7.9) at 4 C. The arrows indicated the points at which the buffer was changed. The enzyme was eluted using the same buffer with increasing concentrations of KCl: 1-0.1 M; 2-0.25 M; 3-0.5 M; 4-1.0 M; and 5-30% (w/v) $(NH_4)_2SO_4$. The flow rate was 12 drops/min and 54 drops/fraction were collected.

-●- absorbance at 280 nm

-o- trypsin activity

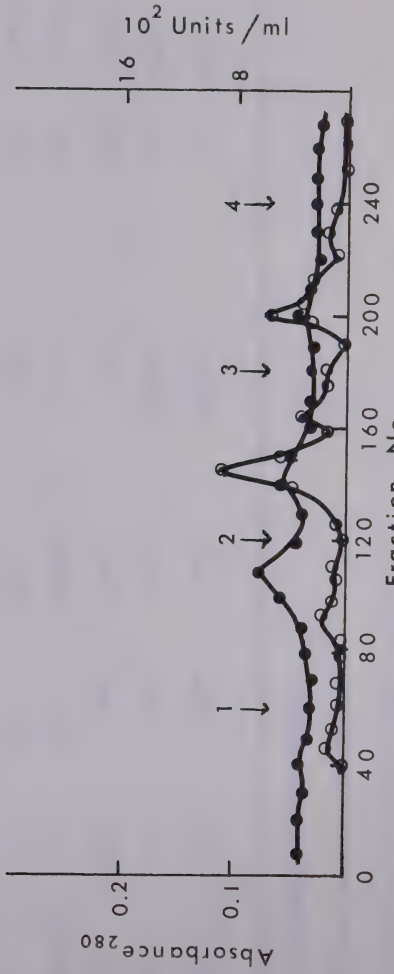

10^2 Units / ml
16
8
Absorbance$_{280}$
0.2
0.1
0
40
80
120
160
200
240
Fraction No.
1
2
3
4

Table I. Purification of *Aedes aegypti* trypsin

Preparation	Volume (ml)	Protein (mg/ml)	Activity [1] (units/ml)	Specific activity (units/mg protein)	Yield (%)	Purification
Crude extract	10	24	10,045	419	100	1
50% SAS ppt[2].	5	12	7,032	587	35	1.39 x
Up to 80% SAS ppt.	5	15	22,099	1,475	110	3.52 x
Sephadex G-100	20.5	0.46	3,014	6,549	61.5	15.68 x
DEAE-cellulose[3]	63	0.014	563	40,180	35.5	95.8 x
DEAE-cellulose[4]	19.8	0.008	482	60,270	9.5	144.2 x

1 1 unit = 1 µM BAEE hydrolyzed/min.

2 SAS = saturated ammonium sulphate.

3 After 0.05 M Tris-0.25 M KCl buffer (pH 7.9).

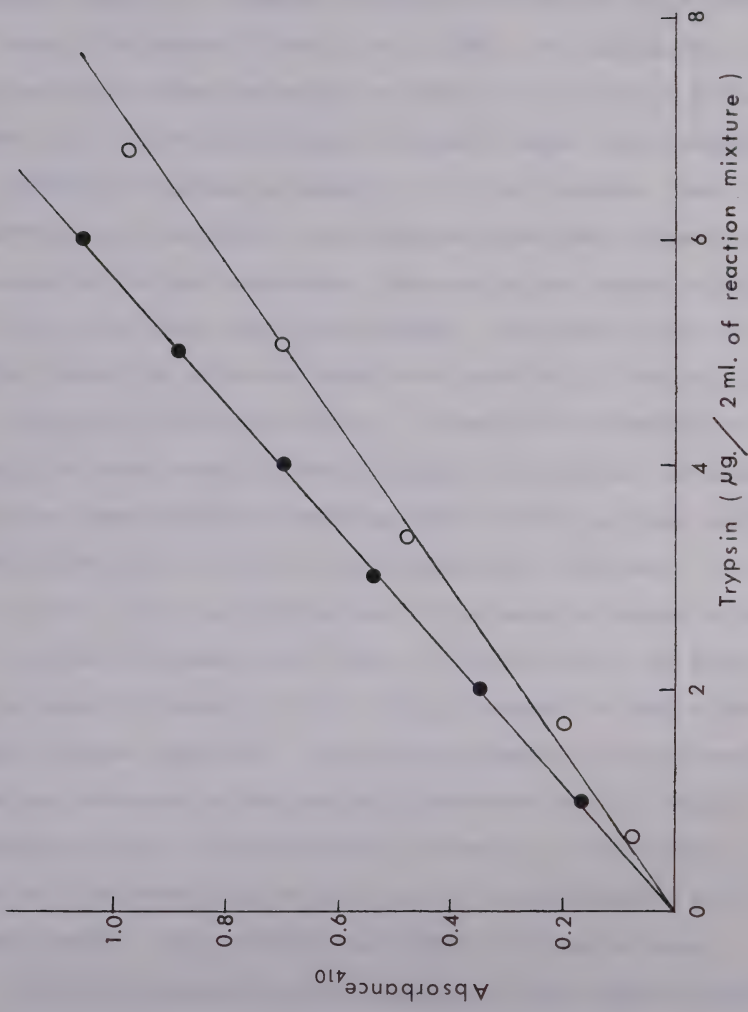
4 After 0.05 M Tris-0.5 M KCl buffer (pH 7.9).

Figure 3. Standard curve for *Aedes aegypti* trypsin and bovine trypsin hydrolysis of BAPNA (3 mM).

The Tris buffer (0.05 M, pH 7.9) contained no $CaCl_2$ for *A. aegypti* trypsin assay, but contained 0.054 M $CaCl_2$ for bovine trypsin assay. The reaction was carried out for 20 minutes at 37 C.

-o- *A. aegypti* trypsin

-●- bovine trypsin


Absorbance 410
1.0
0.8
0.6
0.4
0.2
0
2
4
6
8
Trypsin (µg./ 2 ml. of reaction mixture)

with which the mosquito can digest blood from different sources.

The assay procedure for determining the trypsin or bovine trypsin inhibition capacity of seventeen vertebrate sera and one insect haemolymph was based on the method of Bieth *et al.*, (1968). For each assay, 0.2 ml solution of the appropriate enzyme was added to 1.2 ml of 0.05 M Tris buffer (pH 7.9) containing various dilutions of serum. After mixing, the solution was allowed to incubate at 37 C for 6 minutes, then 0.1 ml of BAPNA (0.03 M) was added to each solution, mixed and incubated another 20 minutes at the same temperature. The reaction was stopped by addition of 0.5 ml of 30% (w/v) acetic acid solution. The blank solution was similar, except the enzyme was added after acetic acid. Each solution was read against the blank at 410 nm. The method for determining the capacity of bovine serum is shown in Figure 4 to illustrate the method. The points representing the remaining enzyme activity on Y-axis and the serum concentration on the X-axis were connected to both axes. The value on the Y-axis was referred back to the amount of enzyme inhibited (7.5 µg) from the standard curve (Fig. 3) and the value on the X-axis was the amount of serum (1.7 µl or 76.65 µg) required to inhibit this amount of enzyme completely. The inhibition capacity of serum from each animal was determined in this way and is expressed as µg of trypsin inhibited/µg (or µl) of serum (Tables II A and B). The inhibition capacity is relatively high in birds and low in elasmobranch , with mammals, reptile, frog, teleosts, and insect in the middle range.

In order to separate the serum inhibitors of each animal, diluted vertebrate sera and the haemolymph of *Periplaneta americana* were put separately onto a calibrated Sephadex G-200 gel column (1.25 x 102 cm)

Figure 4. Inhibition of *Aedes aegypti* trypsin (13.52 µg/ml) hydrolysis of BAPNA (3 mM) by varying amounts of bovine serum in 0.05 M Tris buffer (pH 7.9) for 20 minutes at 37 C. The inhibition capacity was given as µg of enzyme inhibited/µl or µg of seru

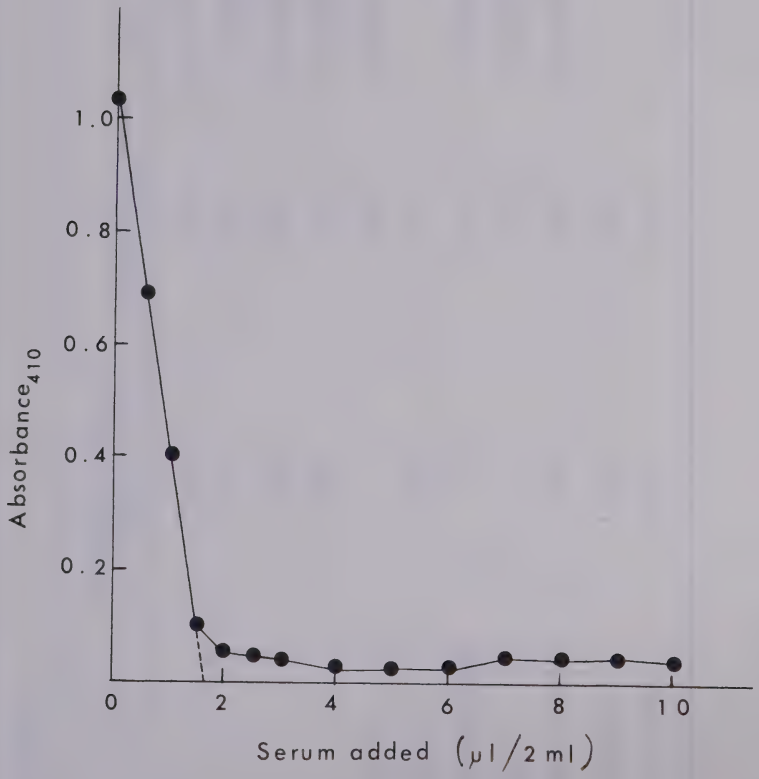

1.0
0.8
0.6
0.4
0.2
Absorbance410
0
2
4
6
8
10
Serum added (µl/2 ml)

Table II A. Inhibition capacity of homeothermic animal sera and molecular weight estimates for the inhibitors

Source	Inhibition capacity µg trypsin/µl serum	µg trypsin/µg serum	Approximately mol. wt.		
Human, *Homo sapiens*	1.62	0.04	≥160,000	100,000	41,700
Cow, *Bos taurus*	4.41	0.10	≥160,000		43,500
Pig, *Sus scrofa*	3.56	0.06	≥160,000		47,900
Sheep, *Ovis aries*	5.36	0.06	≥160,000		47,900
Horse, **Equus caballus*		0.05	≥160,000		47,900
Rat, *Rattus norvegicus*	3.38	0.07			66,100
Rabbit, **Oryctolagus cuniculus*		0.03	≥160,000		39,800
Dog, **Canis familiaris*		0.02	≥160,000		41,700
Elk, *Cervus canadensis nelsoni*	2.76	0.04	≥160,000		39,800
Chicken, *Gallus domesticus*	18.65	0.21			57,500
Turkey, *Meleagris gallopavo*	13.15	0.10			50,100

*lyophilized serum.

Table II B. Inhibition capacity of poikilothermic animal sera and molecular weight estimates for the inhibitors

Source	Inhibition capacity µg trypsin/µl serum	µg trypsin/µg serum	Approximately mol. wt.		
Turtle, *Chrysemys picta*	1.20	0.04	≥160,000	107,000	41,700
Frog, *Rana pipiens*	1.64	0.10	≥160,000	95,500	43,500
Pike, *Esox lucius*	4.31	0.17		77,600	41,700
White fish, *Coregonus clupeaformis*	1.56	0.05	Not determined		
Rockfish, *Sebastodes caurinus*	0.47	0.02	Not determined		
Dogfish, *Squalus acanthias*	0.22	0.01			31,800
Cockroach, *Periplaneta americana*	1.21	0.04			≤11,500

which was previously equilibrated with 0.05 M Tris buffer (pH 7.9) containing 0.1 M KCl. The flow rate and the eluted volume of each fraction were the same as that of enzyme purification. The inhibitor peak and its molecular weight were determined by the method described above. Bovine serum, contained two inhibitors (with approximate molecular weights of 43,500 and >160,000) which inhibit both trypsin and bovine trypsin (Fig. 5). The sera of 16 species have been fractionated and assayed in this manner and the estimated molecular weights of their trypsin inhibitors are presented in Tables II A and B. The results indicate that most animals have more than one trypsin inhibitor in their blood. Therefore, the separation and the characterization of these inhibitors are needed for one to be able to study the interaction of the mosquito trypsin with its serum inhibitors. The two bovine inhibitors have been selected for further study.

3. Purification and properties of inhibitors from bovine serum

a. Purification of inhibitor I

Inhibitor I, in this study, refers to an inhibitor which is associated with the bovine α_1-globulin fraction. The purification procedures for this inhibitor followed the method of Wu and Laskowski (1960). Bovine serum protein which precipitated in 51 to 65% SAS was dissolved in 5 mM sodium phosphate buffer (pH 7) and dialyzed against the same buffer overnight at 4 C. It was then pumped onto a DEAE-cellulose column (2.5 x 18 cm) which was previously equilibrated with the same buffer. The proteins were eluted by increasing the buffer ionic strength and lowering its pH value (Fig. 6). The flow rate and the eluted volume of each fraction are the same as described in enzyme purification. The protein peaks and their protease

Figure 5. Sephadex G-200 chromatography of a 5 ml sample (3.7 mg/ml) of diluted bovine serum.

The column (1.25 x 102 cm) was equilibrated with 0.05 M Tris-0.1 M KCl (pH 7.9) at 4 C. The flow rate was 4 drops/m and 15 drops/fraction were collected.

-●- absorbance at 280 nm

-o- inhibition capacity against *Aedes aegypti* trypsin

-▲- inhibition capacity against bovine trypsin

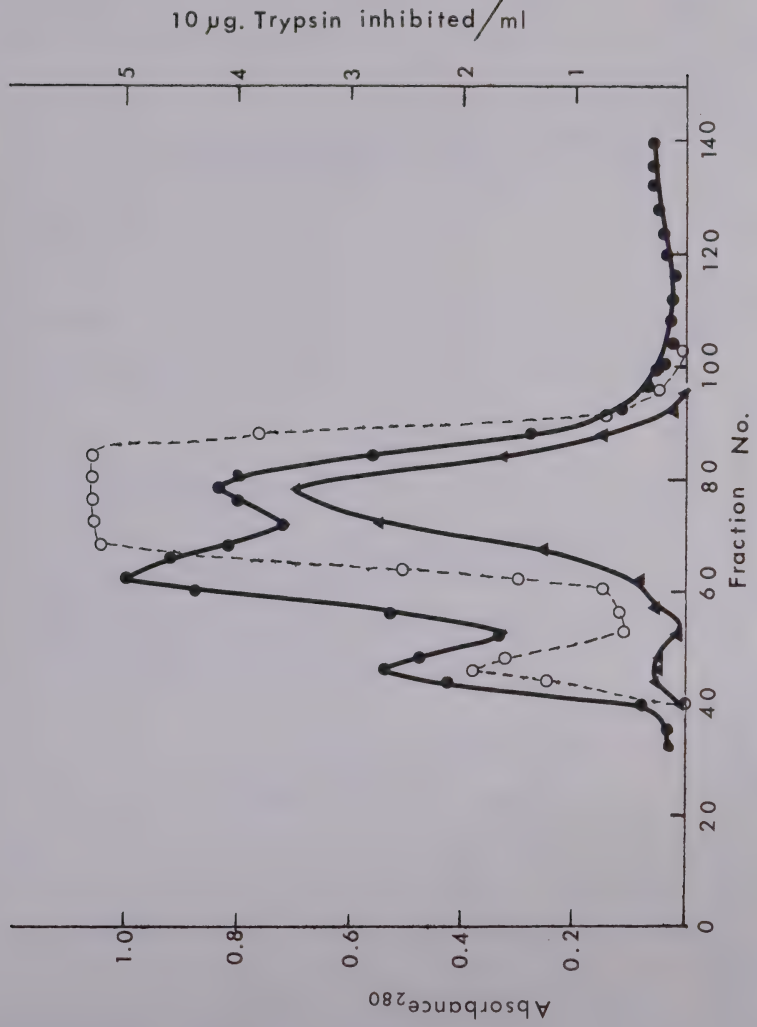
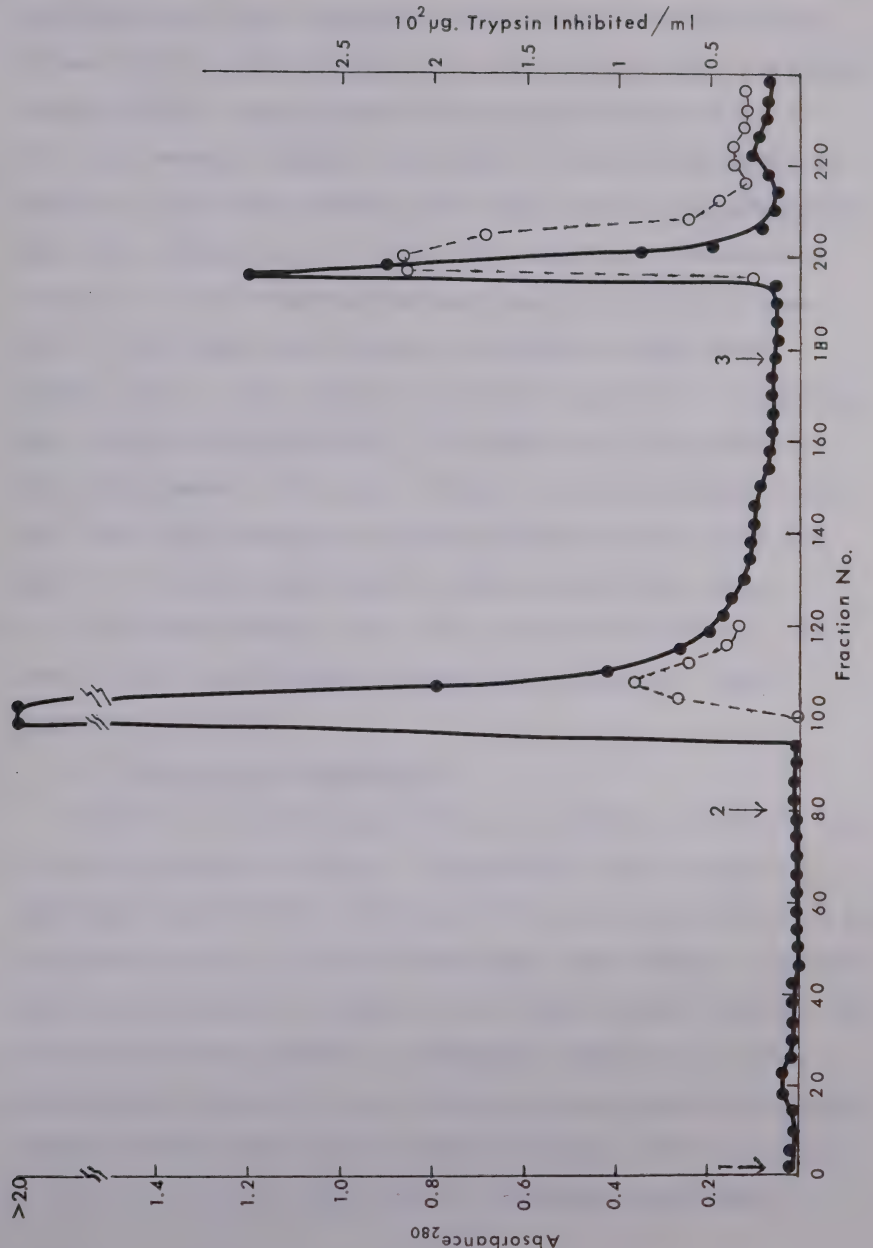

10 µg. Trypsin inhibited/ml
5
4
3
2
1
Absorbance280
1.0
0.8
0.6
0.4
0.2
0
20
40
60
80
100
120
140
Fraction No.

Figure 6. DEAE-cellulose chromatography of 25.5 ml solution (9.5 mg/m
of the 0.51 to 0.65 SAS fraction (Table 3, Step 3) of bovin
serum.

The column (2.5 x 18 cm) was equilibrated with 0.005 M sodi
phosphate buffer (pH 7.0) at 4 C. The flow rate was 12 dro
min and 54 drops/fraction were collected. The arrows indic
the points at which the concentration and the pH of buffer
changed: 1-0.005 M, pH 7; 2-0.05 M, pH 6.5; 3-0.5 M, pH 5.

-●- absorbance at 280 nm

-o- trypsin inhibition capacity


10^2 µg. Trypsin Inhibited/ml
2.5
2
1.5
1
0.5
>20
1.4
1.2
1.0
0.8
0.6
0.4
0.2
Absorbance$_{280}$
1
2
3
0
20
40
60
80
100
120
140
160
180
200
220
Fraction No.

inhibition capacity were determined by the procedures described above. The peak with the greatest amount of trypsin inhibitor (the 0.5 M sodium phosphate buffer) from the previous step was precipitated in 22.6 to 26.1% (w/v) ammonium sulphate solution (pH 6.5) and the precipitate was dissolved in 5 mM sodium acetate buffer (pH 5) and dialyzed against the same buffer overnight at 4 C. It was then pumped onto a CM-cellulose column (2.5 x 18 cm) which was previously equilibrated with the same buffer. The proteins were eluted by increasing the buffer ionic strength (Fig. 7). The inhibitor was eluted mainly in the first protein peak (5 mM sodium acetate buffer). The middle part of this peak was pooled and adjusted to pH 8 with 5 N NH_4OH. It was stored frozen until use. Some of this fraction was dialyzed against distilled water overnight at 4 C and was lyophilized in a freeze dryer (VirTis, model No. 10-145 MR-BA, Gardiner, N.Y., U.S.A.) and then stored frozen. The overall result of purification is summarized in Table III. Three preparations were made.

b. Purification of inhibitor II

Inhibitor II, in this study, refers to an inhibitor associated with the bovine α_2-globulin fraction. This inhibitor is able to form a complex with trypsin which retained most of its esteratic activity but had low protease activity. It will be shown later that inhibitor II protects trypsin from inhibition by inhibitor I and soybean trypsin inhibitor. Use has been made of this property in assaying for inhibitor II. During purification of inhibitor II, its activity was determined as the trypsin-inhibitor esterase (TIE) activity (method of Ganrot, 1966 d) as follows. To 1.2 ml of 0.05 M Tris buffer (pH 7.9) containing proper amount of

Figure 7. CM-cellulose chromatography of an active bovine inhibitor I fraction from DEAE-cellulose column and further $(NH_4)_2SO_4$ salt fraction.

The column (2.5 x 18 cm) was equilibrated with 5 mM sodium acetate buffer (pH 5) at 4 C. The flow rate was 12 drops/mi and 54 drops/fraction were collected. The arrows indicated points at which the concentration of the buffer was changed: 1-5 mM, pH 5; 2-0.05 M, pH 5.

-●- absorbance at 280 nm

-o- trypsin inhibition capacity

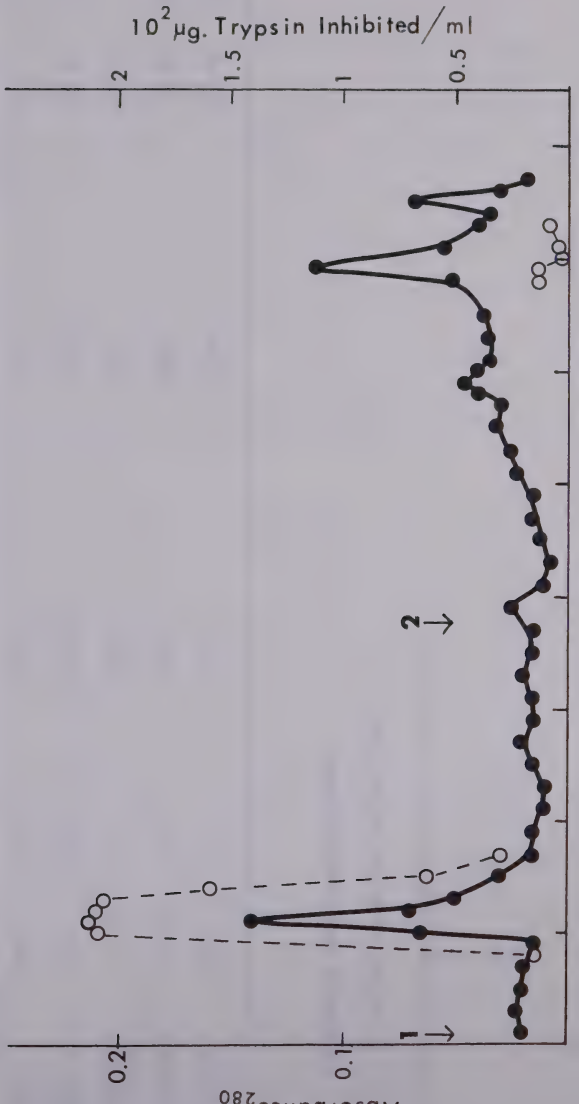

10^2µg. Trypsin Inhibited/ml
2
1.5
1
0.5
Absorbance$_{280}$
0.2
0.1
0
20
40
60
80
100
120
140
160
Fraction No.
1
2

Table III. Purification of Inhibitor I

Preparation	Volume (ml)	Protein (mg/ml)	Inhibition capacity (µg trypsin/ml)	Specific inhibition capacity (µg trypsin/mg protein)	Yield (%)	Purification
Whole serum	600	73.50	7,600	103	100	1
41-90% SAS[1] ppt.	1140	10.50	3,025	288	75.7	2.8 x
51-65% SAS ppt.	26.5	9.5	2,975	313	17.25	3.04 x
DEAE-cellulose[2]	41.7	0.49	1,003	2,040	0.92	19.8 x
CM-cellulose[3]	32	0.09	615	7,240	0.46	70.3 x

1 SAS = saturated ammonium sulphate.

2 after 0.5 M sodium phophate buffer (pH 5.0)

3 after 0.005 M sodium acetate buffer (pH 5.0).

bovine serum or inhibitor II, 0.2 ml of trypsin solution was added. After stirring, the mixture was incubated at 37 C for 6 minutes. Soybean trypsin inhibitor (0.1 ml of 50 µg/ml) or enough inhibitor I to inhibit the free enzyme was added to the mixture, stirred, and incubated for another 6 minutes, and then 0.1 ml of BAPNA solution (0.03 M) was added, mixed and incubated at the same temperature. After 20 minutes, the reaction was stopped by addition of 0.5 ml of acetic acid (30%). A blank sample was made by the same method except the enzyme was added after the acetic acid. The TIE activity was determined from the difference between the absorbance of the sample and the blank at 410 nm. The TIE activity was expressed as µg of trypsin activity retained/2 ml of reaction mixture.

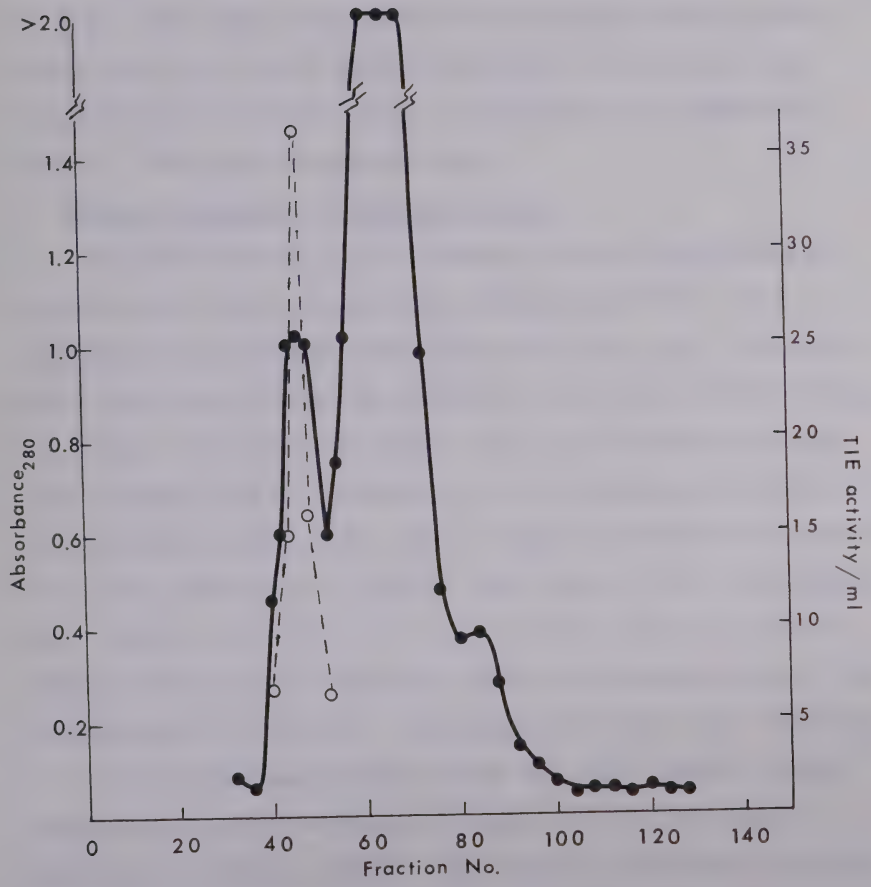
The purification procedure for inhibitor II was modified from Ganrot and Scherstén (1967). To each 250 ml of bovine serum was added 5 ml of 10% (w/v) dextran sulphate and 25 ml $CaCl_2$ (0.1 M); after stirring and centrifuging, the lower density lipoprotein was precipitated and removed. The supernatant was diluted to one liter with 0.1 M phosphate buffer (pH 5.5). The excess Ca^{+2} was precipitated after centrifugation. The supernatant was brought to 0.28 SAS, centrifuged, and the precipitate discarded. The supernatant was brought to 0.40 SAS and the precipitate was separated after centrifugation and dissolved in 10 ml of 0.05 M Tris buffer (pH 7.9) containing 0.1 M KCl. It was dialyzed against the same buffer overnight at 4 C. Each 5 ml of this solution was put onto a Sephadex G-200 gel column (1.25 x 102 cm) (Fig. 8) which was previously equilibrated with the same buffer. The samples contained most of the TIE activity were pooled and dialyzed against 0.05 M Tris buffer (pH 7.9) overnight at 4 C. It was

Figure 8. Sephadex G-200 chromatography of a 5 ml sample (106 mg/ml) of 0.28 to 0.40 SAS fraction of bovine serum.

The column (1.25 x 102 cm) was equilibrated with 0.05 M Tris-0.1 M KCl (pH 7.9) at 4 C. The flow rate was 4 drops/min and 15 drops/fraction were collected.

-●- absorbance at 280 nm

-o- TIE activity


>2.0
1.4
1.2
1.0
0.8
0.6
0.4
0.2
Absorbance280
35
30
25
20
15
10
5
TIE activity/ml
0
20
40
60
80
100
120
140
Fraction No.

pumped onto a DEAE-cellulose column (2.5 x 18 cm) which was previously equilibrated with the same buffer. The proteins were eluted by increasing the KCl concentraction from 0.06 M to 0.15 M in the same buffer (Fig. 9). The TIE activity peaks were eluted after addition of 0.1 M KCl. The samples with most TIE activity were pooled and stored frozen until use. Some of the purified protein was lyophilized and stored frozen. The overall results of purification are summarized in Table IV. Three purifications were made.

c. Molecular properties of inhibitor I and II

Purified inhibitors I and II were each found as single bands on electrophoresis located in positions corresponding to the α_1 - and α_2-globulin region of bovine serum respectively (Fig. 10). The electromotive force was 250 volts, the current was 6 ma, and the time of running was 8 hours. The protein was stained red by the fixative-dye solution which contained 0.2% (w/v) ponceau-S, 3% (w/v) trichloroacetic acid, and 3% (w/v) sulfosalicyclic acid. The electrophoretic mobilities of inhibitors I and II were calculated as -5.80×10^{-5} and -5.21×10^{-5} in 0.06 M barbital buffer (pH 8.6), and -1.30×10^{-5} and -1.23×10^{-5} $cm^2/v/sec$ in 0.05 M phosphate buffer (pH 7) respectively (Schultze and Heremans, 1966). The molecular weight of inhibitor I was estimated as 95,500 after recolumning in a calibrated Sephadex G-100 gel column (Fig. 11). However, another estimate for the molecular weight of inhibitor I is 43,500 (Fig. 5). I think that the purified inhibitor I may exist as a dimer form in the gel column, so its molecular weight is given as 43,500. The molecular weight of inhibitor II could not be estimated since it was excluded from both Sephadex G-100 and G-200 gel columns. I assume that the inhibitor II has a

Figure 9. DEAE-cellulose chromatography of a 24.5 ml sample (10 mg/ml) from Sephadex G-200 column.

The column (2.5 x 18 cm) was equilibrated with 0.05 M Tris buffer (pH 7.9) at 4 C. The arrows indicated the points at which the buffer was changed. The inhibitor was eluted using the same buffer with increasing KCl concentration: 1-0.06 M; 2-0.1 M; 3-0.15 M. The flow rate was 12 drops/min and 54 drops/fraction were collected.

-●- absorbance at 280 nm

-o- TIE activity

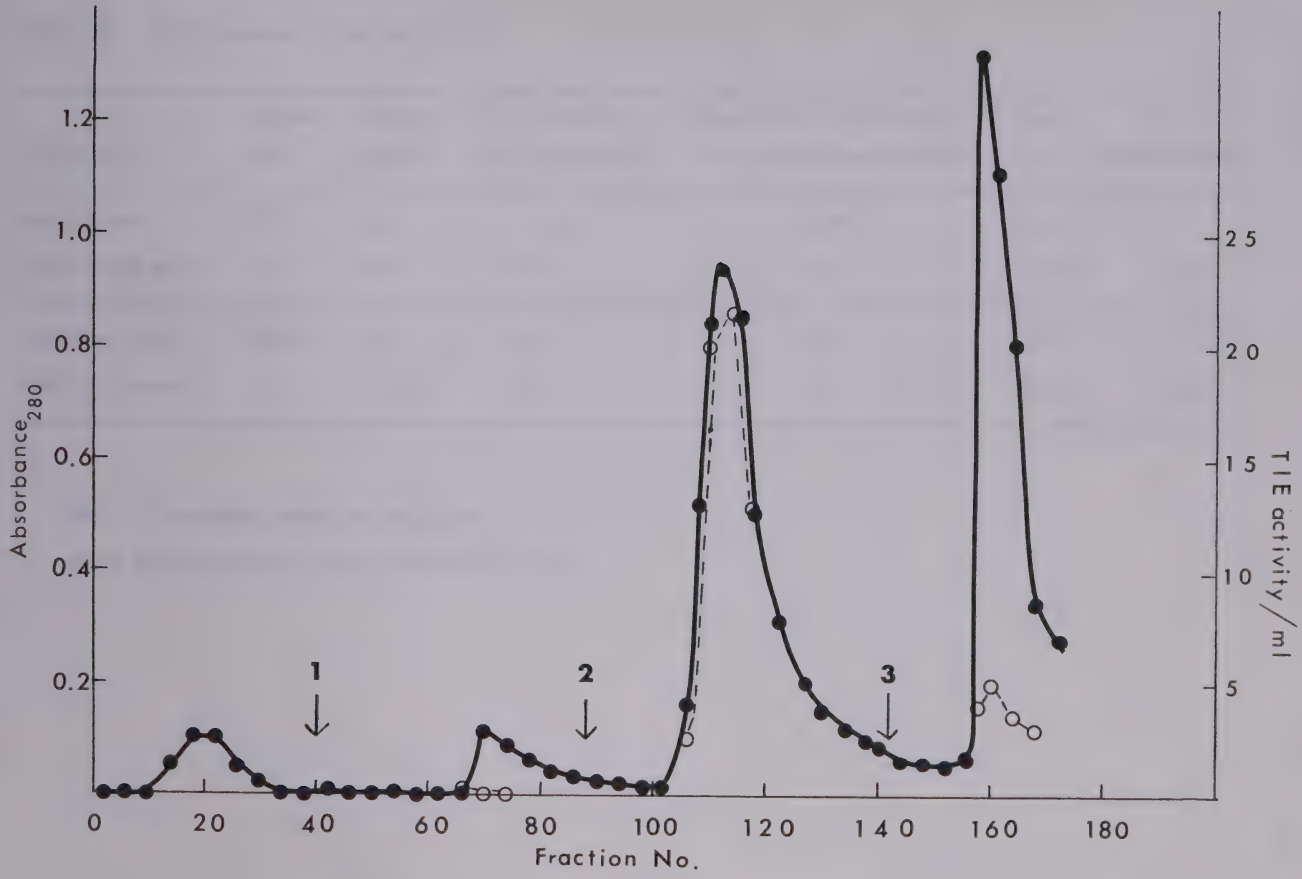

Absorbance280
1.2
1.0
0.8
0.6
0.4
0.2
1
2
3
0
20
40
60
80
100
120
140
160
180
Fraction No.
25
20
15
10
5
TIE activity/ml

Table IV. Purification of inhibitor II

Preparation	Volume (ml)	Protein (mg/ml)	TIE activity (µg trypsin/ml)	Specific TIE activity (µg trypsin/mg protein)	Yield (%)	Purification
Whole serum	250	112	4.5	0.04	100	1
28-40% SAS ppt.[1]	10	106	24	0.23	21.35	5.5 x
Sephadex G-200	166.6	10	64	6.4	100	1
DEAE-cellulose[2]	233	1.08	28	25.9	61.13	4.5 x

[1] SAS = saturated ammonium sulphate.

[2] after 0.05 M Tris-0.1 M KCl buffer (pH 7.9).

Figure 10. Diagrams of protein separation obtained by electrophoresis o
bovine serum, inhibitor I, and inhibitor II.

The electrophoresis was carried out on cellulose acetate mem
in 0.06 M barbital buffer (pH 8.6), 250 volts, 6 ma for 8 ho
at 4 C.

A. bovine serum

B. inhibitor I

C. inhibitor II

1. albumin

2. α_1-globulin

3. α_2-globulin

4. β-globulin

5. γ-globulin

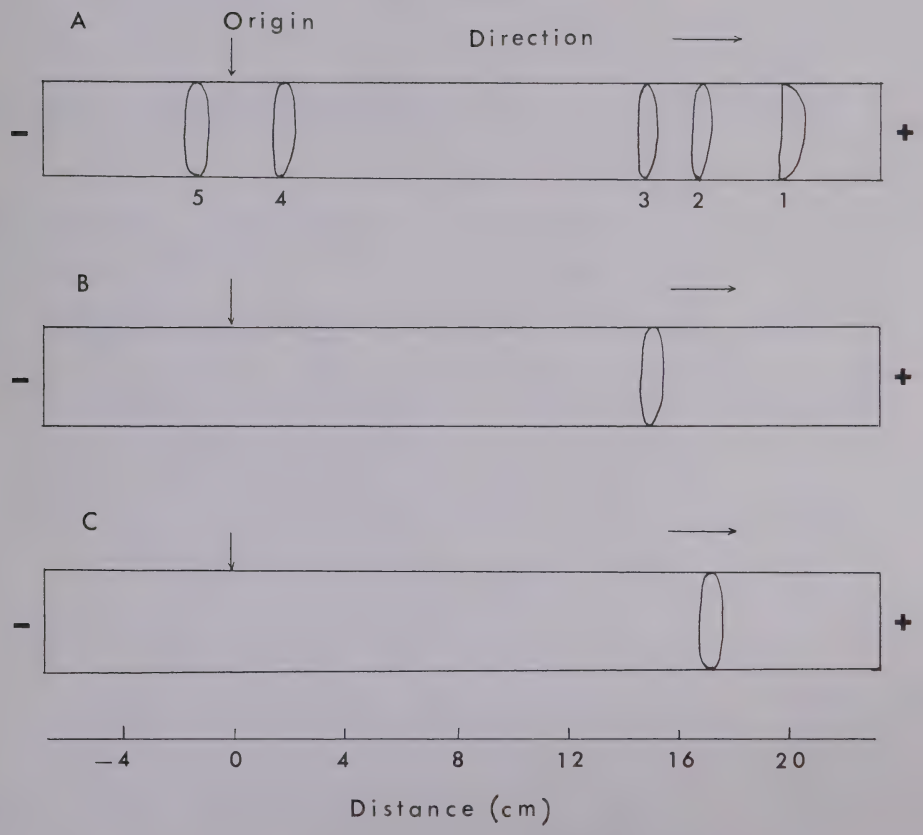

A
Origin
Direction
−
+
5
4
3
2
1
B
−
+
C
−
+
−4
0
4
8
12
16
20
Distance (cm)

Figure 11. Estimation of the molecular weights of proteins by Sephadex g
filtration.

A. G-200 gel column; B. G-100 gel column. Both columns wer
equilibrated with 0.05 M Tris-0.1 M KCl buffer (pH 7.9) at 4
The flow rate was 4 drops/min and 15 drops/fraction were coll

The pure proteins (5 mg/ml) used for standardization were:

△ - bovine albumin (dimer); molecular weight = 134,000

▲ - bovine albumin (monomer); molecular weight = 67,000

□ - bovine hemoglobin; molecular weight = 64,500

■ - β-lactoglobulin; molecular weight = 36,000

○ - soybean trypsin inhibitor; molecular weight = 21,500

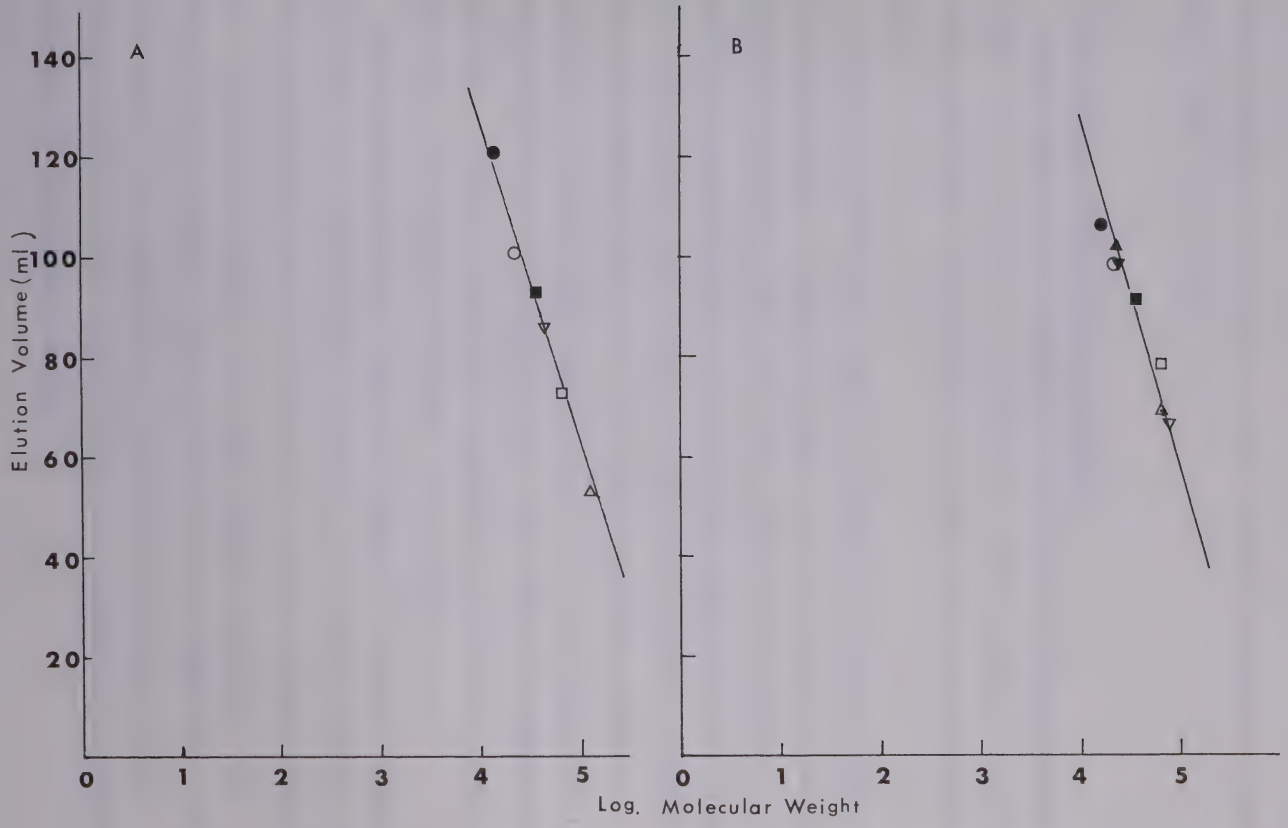
● - α-lactalbumin; molecular weight = 15,500

The proteins for estimation the molecular weight were:

▼ - *Aedes aegypti* trypsin (after 0.25 M KCl)

▲ - *Aedes aegypti* trypsin (after 0.5 M KCl)

▽ - inhibitor I


A
B
140
120
100
80
60
40
20
Elution Volume (ml)
0
1
2
3
4
5
Log. Molecular Weight

molecular weight about 1,000,000, the same as that of foetal calf α_2-macroglobulin (Marr *et al.*, 1962).

4. The studies of enzyme-inhibitor complexes

Whole bovine serum non-competitively inhibited the action of trypsin on BAPNA. The K_i is 159.48 ± 4.05 µg/2 ml of reaction mixture. All further studies on the nature of the inhibition were done with purified inhibitors I and II.

Data on the inhibition of trypsin by inhibitor I is given in Figure 12. The points which give the remaining enzymic activity on the Y-axis and the inhibitor concentration on the X-axis were connected as a straight line after the simple regression analysis and the line was extrapolated to the X-axis. The X-axis intercept gives the amount of inhibitor required to completely inhibit the amount of enzyme used. The average inhibition capacity of inhibitor I for trypsin (three experiments) is 1.73 ± 0.24 (µg/µg) and the average molar ratio of the complex is 3.50 ± 0.49 (moles of enzyme/mole of inhibitor). For bovine trypsin (one experiment), the inhibition capacity of inhibitor I is 0.83 and the molar ratio of the complex is 1.50.

Data on the inhibition of trypsin by inhibitor II is given in Figure 13. The points which represent either the remaining total enzymic activity (the free trypsin activity and the TIE activity), or the free trypsin activity only, are connected separately after simple regression analysis. The average inhibition capacity and the molar ratio for the complex are: 0.04 ± 0.004 and 1.86 ± 0.20 respectively if calculated on the basis of total enzymic activity and 0.04 ± 0.003 and 1.97 ± 0.12 respectively if calculated on the basis of free enzymic activity.

Figure 12. Inhibition of trypsin by inhibitor I.

The reaction was carried out in 0.05 M Tris buffer (pH 7.9) for 20 minutes at 37 C.

-●- 0.1 ml enzyme (29.87 µg/ml) used

-▲- 0.15 ml enzyme (29.87 µg/ml) used

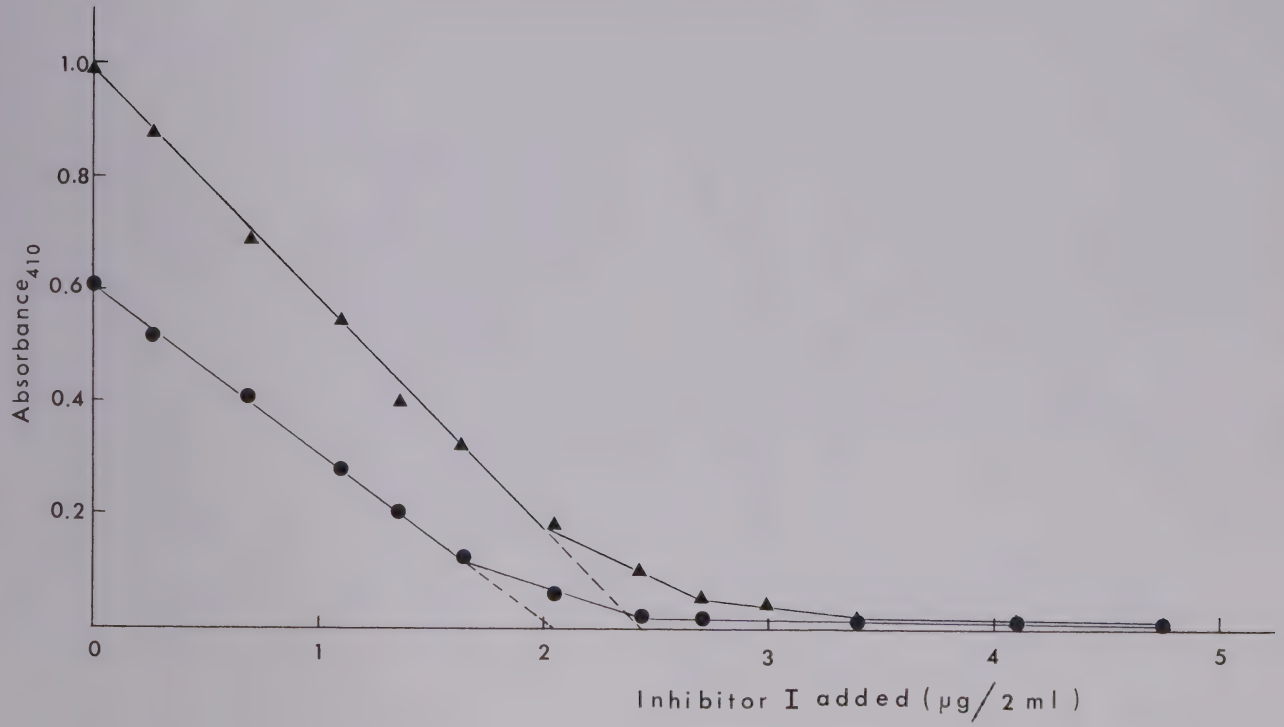

1.0
0.8
0.6
0.4
0.2
Absorbance$_{410}$
0
1
2
3
4
5
Inhibitor I added (µg/2 ml)

Figure 13. Inhibition of trypsin by inhibitor II.

The reaction was carried out in 0.05 M Tris buffer (pH 7.9) for 20 minutes at 37 C. No inhibitor I was added.

total activity:

-o- 0.1 ml enzyme (29.87 µg/ml) used

-△- 0.15 ml enzyme (29.87 µg/ml) used

free trypsin activity:

-●- 0.1 ml enzyme (29.87 µg/ml) used

-▲- 0.15 ml enzyme (29.87 µg/ml) used

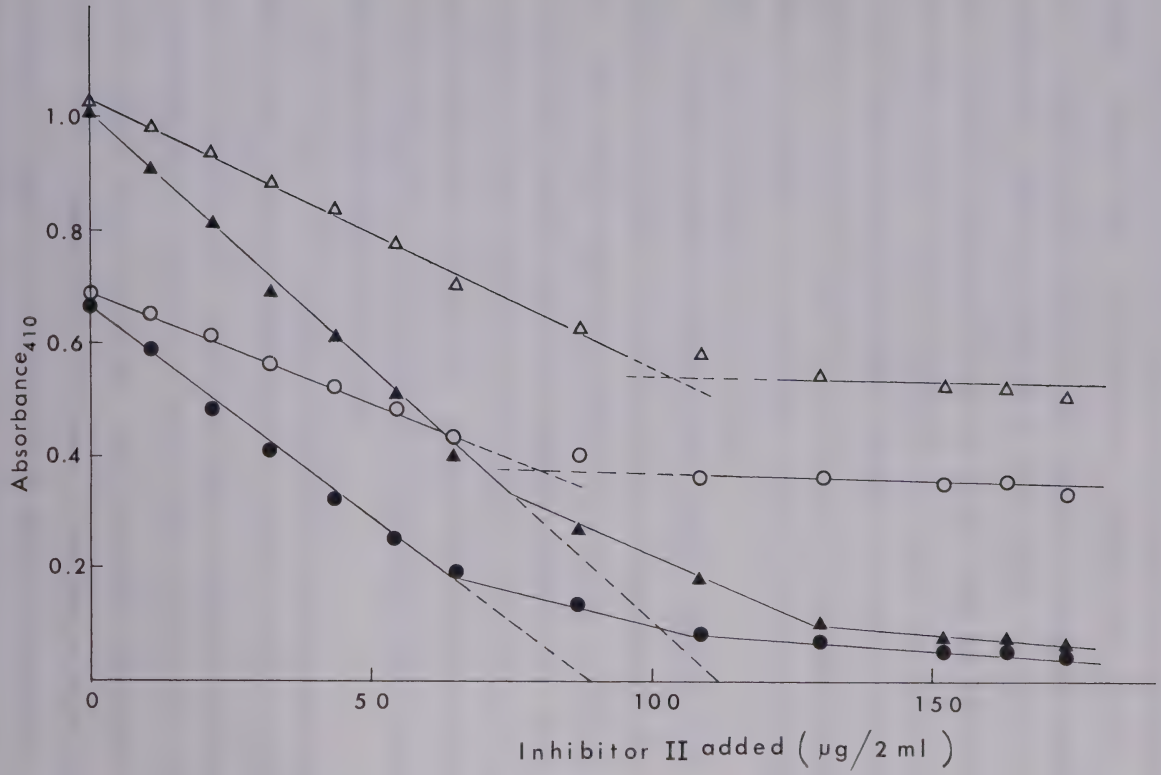

1.0
0.8
0.6
0.4
0.2
Absorbance410
0
50
100
150
Inhibitor II added (µg/2 ml)

Figure 14 gives the TIE activity curve of the trypsin-inhibitor II complex. In this study, 0.1 ml inhibitor I solution (136 μg/ml) was added before adding the BAPNA solution to the mixture of trypsin and inhibitor II. The inhibitor I completely inhibited the free trypsin. The points in Figure 14 give the remaining TIE activity on the Y-axis and inhibitor II concentration on the X-axis are connected after simple regression analysis. The lines intercept at a point which indicates the amount of inhibitor II required to saturate the amount of trypsin used. The average inhibition capacity of inhibitor II for the trypsin is 0.04 ± 0.01 and the average molar ratio of the complex is 1.72 ± 0.45.

By using a Sephadex G-100 gel column, trypsin, inhibitor I and inhibitor II were found at fractions 82, 50 and 40 respectively (Fig. 15 A, B, C). The complex of trypsin and inhibitor I was found at fraction 50 and had neither enzymic activity nor inhibition capacity (Fig. 15 D), while the complex of trypsin-inhibitor II was found at fraction 40 and it retained some esteratic activity (Fig. 15 E). The esteratic activity of the complex of trypsin and inhibitor II was found also in association with the protein complex after electrophoresis (Fig. 16).

Free trypsin and its complex with inhibitor II were compared with respect to the K_m values for BAPNA (Fig. 17) and the effects of pH upon enzymic activity (Fig. 18). Trypsin has a lower affinity for BAPNA than its complex form with inhibitor II. However, their esteratic activities both have similar pH-activity curves. The K_m for BAPNA is lower than for denatured bovine hemoglobin, but the number of substrate molecules bound per molecule of trypsin is one when either of these two substrates is used (Table V). One molecule of BAPNA is also bound by one molecule of trypsin-inhibitor II complex (Table V).

Figure 14. Esteratic activity of the complex of trypsin and inhibitor II

The reaction was carried out in 0.05 M Tris buffer (pH 7.9) for 20 minutes at 37 C; 0.1 ml inhibitor I (136 µg/ml) was ad before the BAPNA.

TIE activity:

—●— 0.1 ml enzyme (29.87 µg/ml) used

—▲— 0.15 ml enzyme (29.87 µg/ml) used

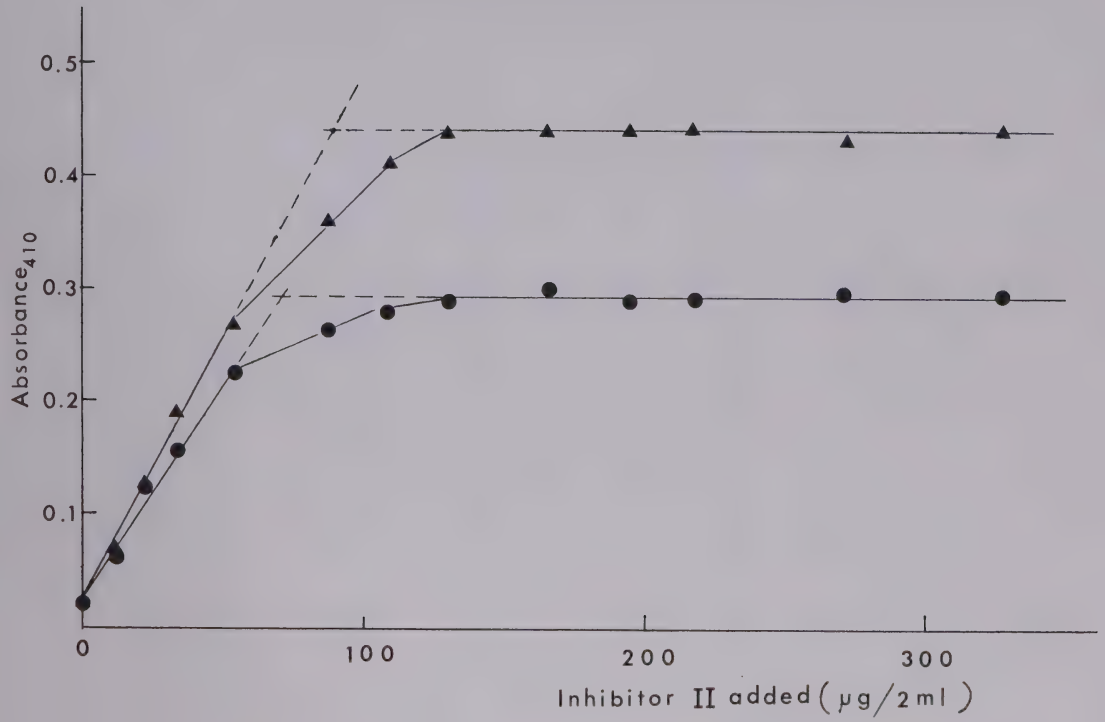

0.5
0.4
0.3
0.2
0.1
Absorbance410
0
100
200
300
Inhibitor II added (μg/2ml)

Figure 15. Sephadex G-100 chromatography of trypsin, inhibitor I and inhibitor II.

The column (1.25 x 102 cm) was equilibrated with 0.05 Tris-0
KCl (pH 7.9) at 4 C. The flow rate was 4 drops/min and 15 drops/fraction were collected.

A. 6 ml trypsin (0.03 mg/ml)

B. 6 ml inhibitor I (0.135 mg/ml)

C. 6 ml inhibitor II (1.111 mg/ml)

D. 6 ml trypsin and 1 ml inhibitor I. The mixture was preincubated 6 minutes at 37 C and stored overnight at 4

E. 3 ml trypsin and 3 ml inhibitor II. The mixture was preincubated 6 minutes at 37 C and stored overnight at 4

-●- absorbance at 280 nm

-o- trypsin activity

-Δ- % of inhibition.

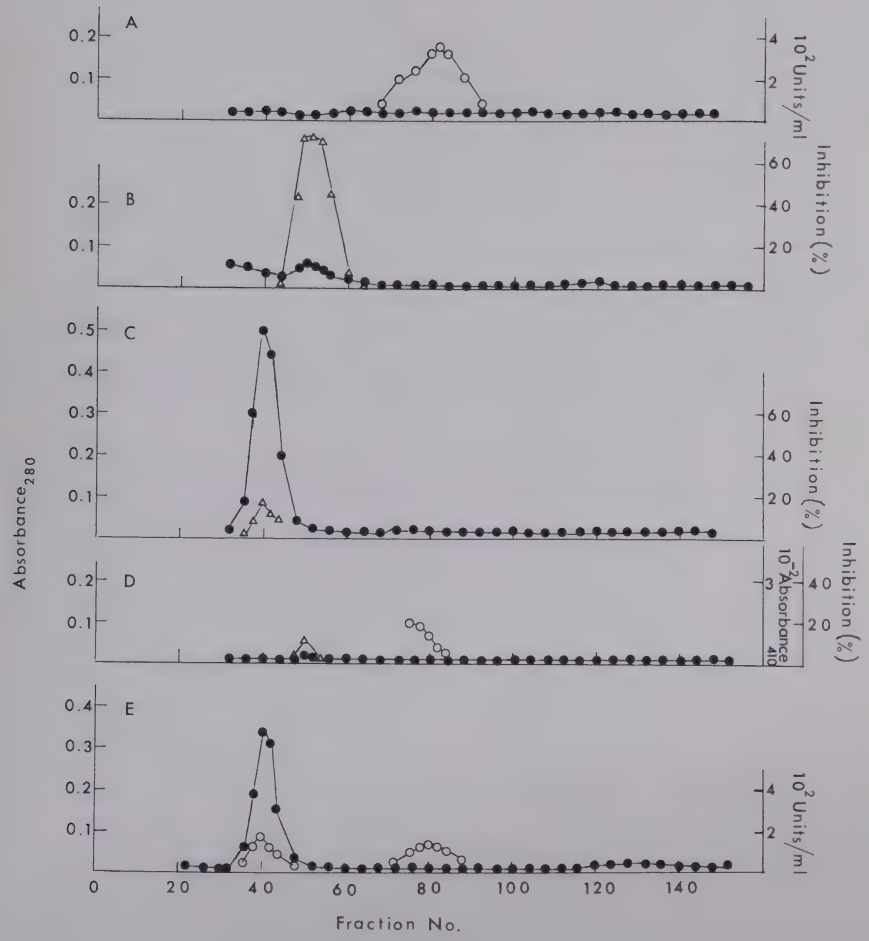

A
B
C
D
E
Absorbance280
10²Units/ml
Inhibition (%)
10⁻²Absorbance410
0.1
0.2
0.3
0.4
0.5
2
4
20
40
60
0
20
40
60
80
100
120
140
Fraction No.

Figure 16. The demonstration of complex formation between isolated inhib
II and trypsin by cellulose acetate membrane electrophoresis.

The electrophoresis was carried out in 0.06 M barbital buffer
(pH 8.6), 310 volts, 8 ma for 5 hours at 4 C. The sample u
was from Fig. 15 E. The esteratic activity was measured afte
six hours of incubation at 37 C and it accompanied the inhibi
peak.

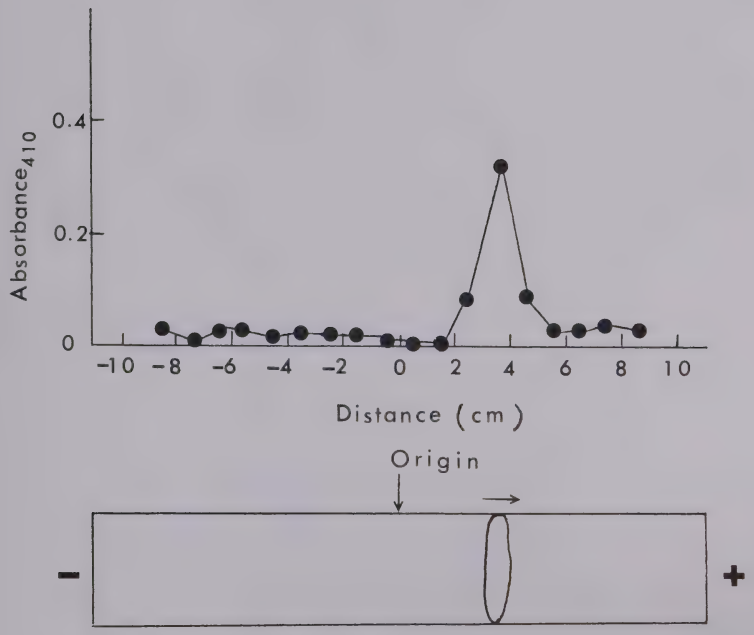

Absorbance410
0.4
0.2
0
-10 -8 -6 -4 -2 0 2 4 6 8 10
Distance (cm)
Origin
−
+

Figure 17. Effect of BAPNA concentration upon activity of free trypsin and trypsin-inhibitor II complex.

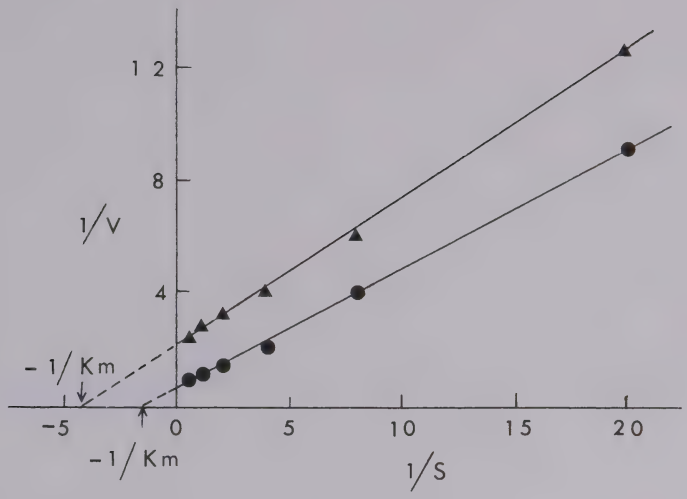
The reaction was carried out in 0.05 M Tris buffer (pH 7.9) at 37 C.

-●- trypsin

-▲- trypsin-inhibitor II complex

S = BAPNA concentration (mM)

V = reaction velocity (absorbance at 410 nm)


1 2
8
1/V
4
- 1/ Km
-5
0
5
10
15
20
-1/Km
1/S

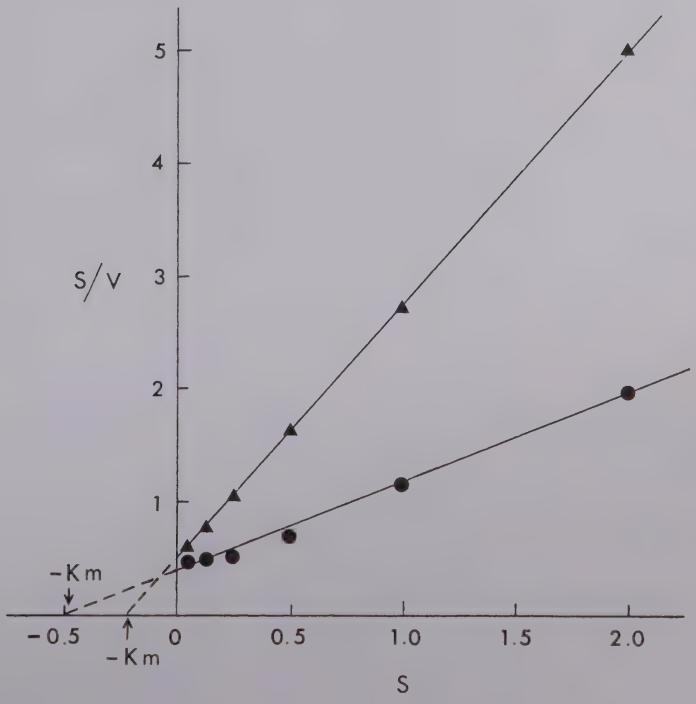

5
4
S/V
3
2
1
-Km
-0.5
0
0.5
1.0
1.5
2.0
-Km
S

Figure 18. Effect of pH upon activity of free trypsin and trypsin-inhib
complex.

The reaction was carried out in the following buffers at 37
for 20 minutes.

0.05 M sodium acetate buffer (pH 4; 5)

0.05 M sodium phosphate buffer (pH 6; 7)

0.05 M Tris buffer (pH 8; 9)

0.05 M sodium bicarbonate buffer (pH 10)

-●- free trypsin

-▲- trypsin-inhibitor II complex

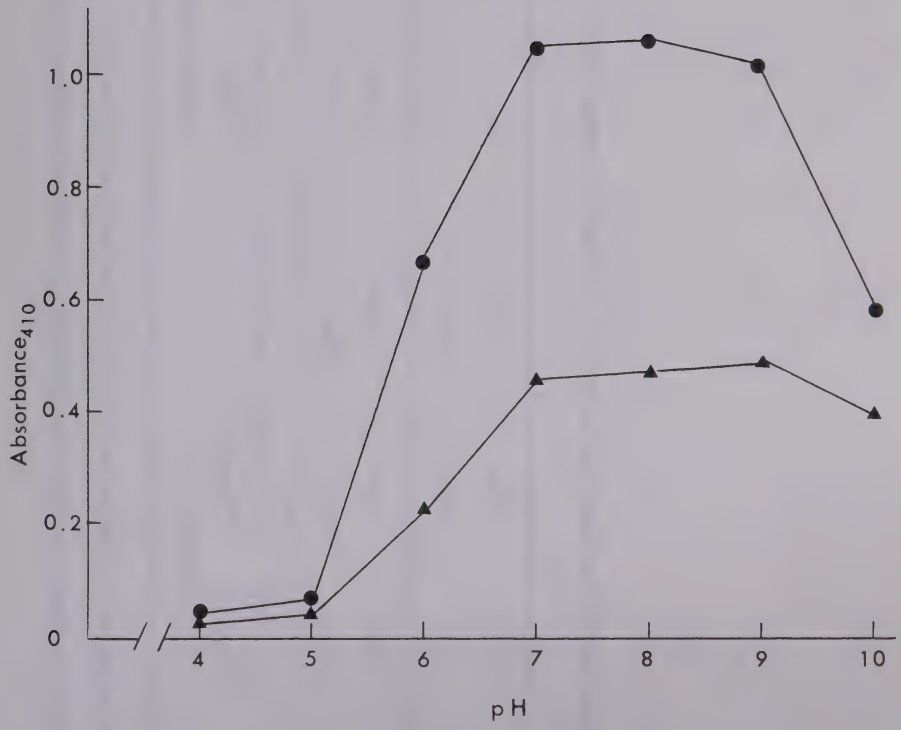

1.0
0.8
0.6
0.4
0.2
0
Absorbance410
4
5
6
7
8
9
10
pH

Table V. The K_m and the number of substrate molecules bound per molecule of trypsin and of trypsin-inhibitor II complex in 0.05 M Tris buffer (pH 7.9) at 37 C

Substrate	Trypsin			Trypsin-inhibitor II complex		
	K_m (mM) (S/V vs S)	K_m (mM) (1/V vs 1/S)	No. of sites[1]	K_m (mM) (S/V vs S)	K_m (mM) (1/V vs 1/S)	No. of sites[1]
BAPNA	0.47± 0.01	0.71± 0.01	1.04± 0.004	0.21± 0.007	0.22± 0.03	1.0± 0.02
Denatured bovine hemoglobin	2.24± 0.03	1.95± 0.83	0.99± 0.05	-	-	-

[1] number of substrate molecules bound per molecule of enzyme (or enzyme-inhibitor complex).

The K_i and the type of inhibition were determined by the Dixon plot (Figs. 19 and 20). It appears that both inhibitor I and inhibitor II are competitive inhibitors of trypsin when denatured bovine hemoglobin is used, while they are non-competitive inhibitors when BAPNA is used. However, both are mixed type inhibitors, since the K_m and the V_{max} are altered with increased inhibitor concentration (Table VI). The Hill plot to determine the number of inhibitor molecules bound per molecule of enzyme is shown in Figure 21. Inhibitor I has a lower K_i than the inhibitor II when either denatured bovine hemoglobin or BAPNA are used as substrates, and the number of inhibitor molecules bound per molecule of trypsin are about 1.6 to 1.8 (Table VII).

The temperature effect on the K_i, type of inhibition, and the number of inhibitor molecules bound per molecule of enzyme is given in Table VIII. The K_i increased gradually for both inhibitor I and inhibitor II when the assay temperature was raised from 30 to 44.5 C. The type of inhibition for both inhibitors was competitive at 30 and 34 C, but it became non-competitive at 37 and 44.5 C. The heat of activation (ΔH°) of these inhibitors was determined by van't Hoff plot (Fig. 22). The thermodynamic parameters of enzyme-inhibitor complex and of enzyme-substrate complex are summarized in Table IX.

The esteratic activity of trypsin-inhibitor II complex is not inhibited completely by an excess amount of inhibitor I, soybean trypsin inhibitor, or PMSF, but it is inhibited completely by an excess amount of TLCK. The esteratic activity of trypsin is inhibited completely by inhibitor I, soybean trypsin inhibitor, PMSF, or TLCK, and this enzyme

Figure 19. Determination of K_1 and type of inhibition by inhibitor I a
inhibitor II.

A. inhibitor I; B. inhibitor II. The substrate was denatu
bovine hemoglobin at a concentration of 4% (S1) or 6% (S2).
The trypsin concentration was 9.5 μg/2 ml reaction mixture.
reaction was carried out in 0.05 M Tris buffer (pH 7.9) for
minutes at 37 C.

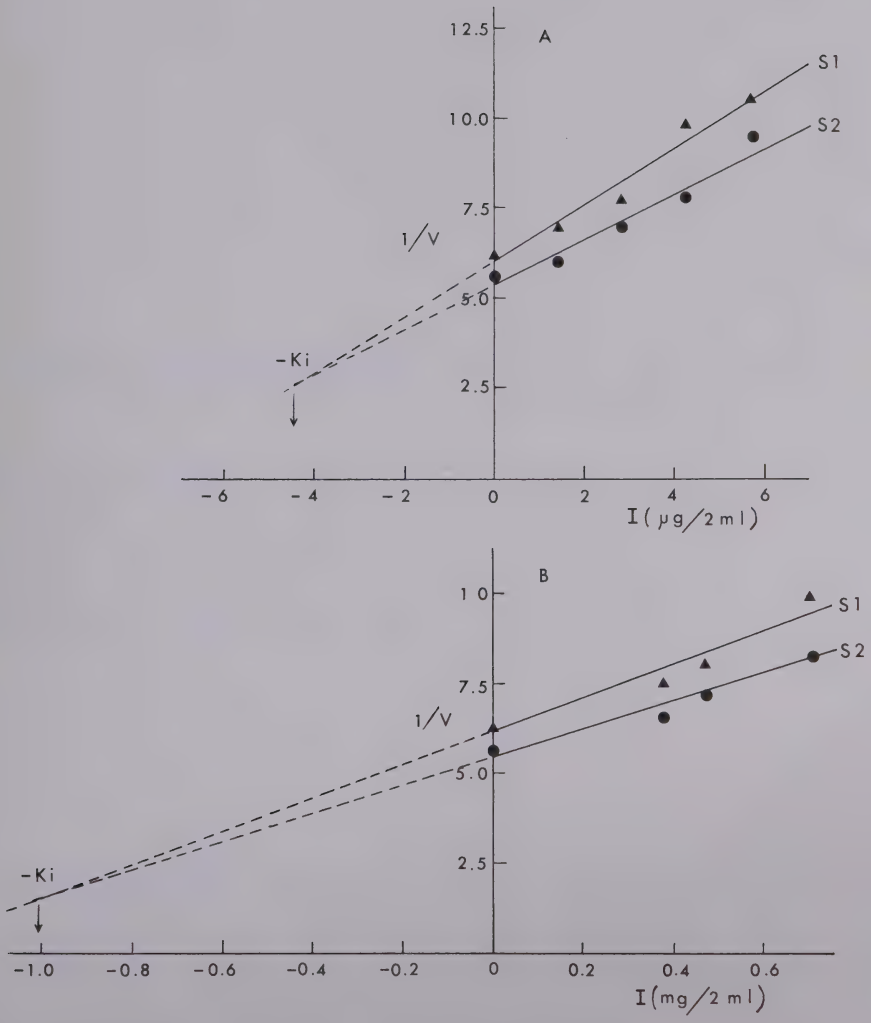
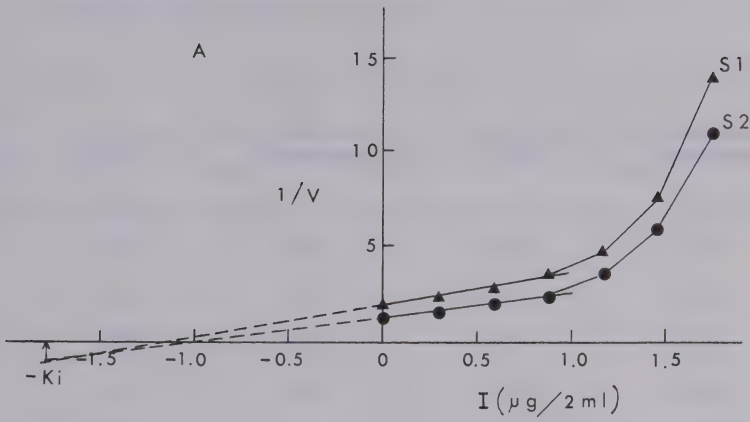

A
12.5
10.0
7.5
5.0
2.5
1/V
S1
S2
-Ki
-6
-4
-2
0
2
4
6
I (µg/2 ml)
B
10
7.5
5.0
2.5
1/V
S1
S2
-Ki
-1.0
-0.8
-0.6
-0.4
-0.2
0
0.2
0.4
0.6
I(mg/2 ml)

Figure 20. Determination of K_i and type of inhibition of inhibitor I and inhibitor II.

A. inhibitor I; B. inhibitor II. The substrate was BAPNA a a concentration of 0.5 mM (S1), or 3 mM (S2). The trypsin concentration was 3.38 µg/2 ml reaction mixture. The reactio was carried out in 0.05 M sodium phosphate buffer (pH 7.9) fo 20 minutes at 37 C.


A
15
10
5
1/V
S1
S2
-1.5
-1.0
-0.5
0
0.5
1.0
1.5
-Ki
I (μg/2ml)

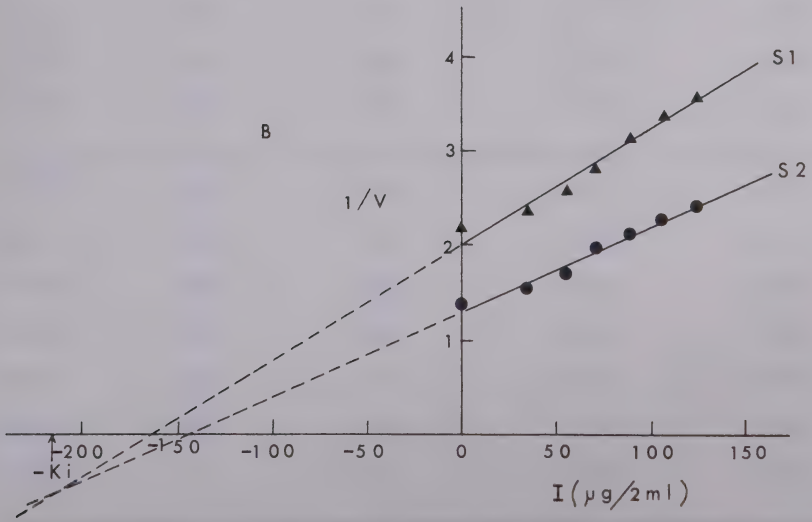

B
4
3
2
1
1/V
S1
S2
-200
-150
-100
-50
0
50
100
150
-Ki
I (μg/2ml)

Table VI. The (S/V vs S) and (1/V vs 1/S) plot to determine the K_m and the V_{max} when either inhibitor I or inhibitor II was used to inhibit the trypsin hydrolysis of BAPNA in 0.05 M sodium phosphate buffer (pH 7.9) at 37 C

Inhibitor	S/V vs S[3]		1/V vs $1/S^3$	
(μg/2 ml)	K_m (mM)	V_{max} (Absorbance$_{410}$)	K_m (mM)	V_{max} (Absorbance$_{410}$)
$\frac{I^1}{0}$	0.31	0.84	0.37	0.87
0.29	0.24	0.70	0.27	0.71
0.58	0.24	0.57	0.28	0.59
0.87	0.23	0.44	0.26	0.45
1.16	0.13	0.28	0.19	0.30
1.45	0.13	0.18	0.19	0.19
1.74	0.19	0.10	0.18	0.10
$\frac{II^2}{0}$	0.37	0.82	0.41	0.84
35.9	0.30	0.71	0.36	0.75
53.85	0.28	0.64	0.34	0.67
71.80	0.23	0.56	0.31	0.59
89.75	0.25	0.51	0.33	0.53
107.70	0.27	0.48	0.34	0.50
125.65	0.27	0.46	0.34	0.58

[1] Inhibitor I

[2] Inhibitor II

[3] BAPNA concentration: 0.5 mM; 1 mM; 3 mM.

Figure 21. The Hill plot, log [(V_o/V) - 1] versus log [I].
The substrate was BAPNA at a concentration of 0.5 mM or 3 mM
The trypsin concentration was 3.38 µg/2 ml reaction mixture.
The slopes of the lines yield the number of inhibitor molecul
bound/molecule of enzyme. A. inhibitor I; B. inhibitor II.

—▲— 0.5 mM BAPNA

—●— 3 mM BAPNA

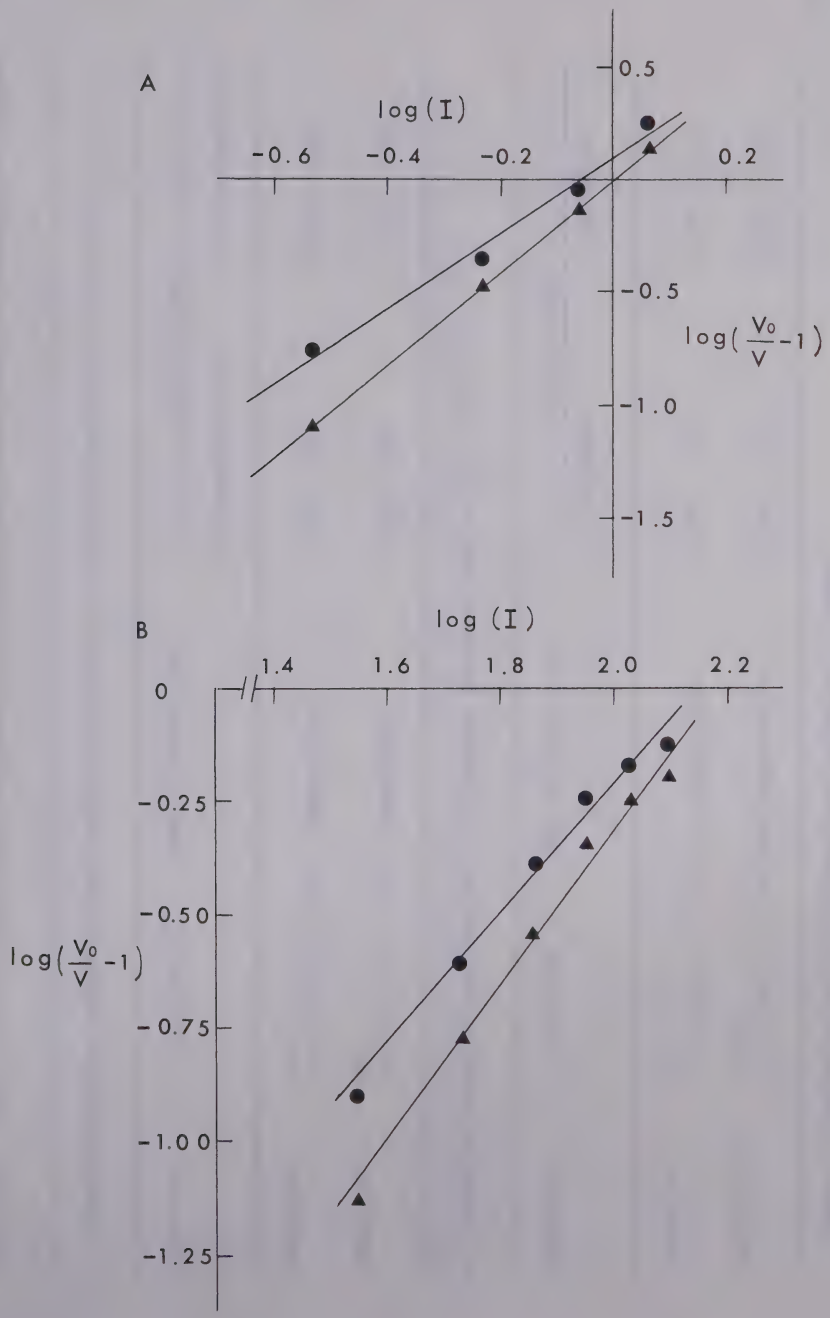

A
log (I)
-0.6
-0.4
-0.2
0.2
0.5
-0.5
-1.0
-1.5
log(Vo/V -1)
B
log (I)
1.4
1.6
1.8
2.0
2.2
0
-0.25
-0.50
-0.75
-1.00
-1.25
log(Vo/V -1)

Table VII. The K_i, type of inhibition, and number of inhibitor molecules bound per molecule of trypsin

A. Denatured bovine hemoglobin was used as substrate in 0.05 M Tris buffer (pH 7.9) at 37 C

Inhibitor	K_i (μg/2 ml)	Type of inhibition	No. of sites[1]	
			4% denatured Hb[2].	6% denatured Hb[2].
Inhibitor I	4.67 (= 5.37×10^{-8} M)	Competitive	1.85 ± 0.17	1.95 ± 0.16
Inhibitor II	1034 (= 61.7×10^{-8} M)	Competitive	1.83 ± 0.06	1.83 ± 0.33

B. BAPNA was used as substrate in 0.05 M sodium phosphate buffer (pH 7.9) at 37 C

Inhibitor	K_i (μg/2 ml)	Type of inhibition	No. of sites[1]	
			0.5 mM BAPNA	3 mM BAPNA
Inhibitor I	1.81 (= 2.08×10^{-8} M)	Non-competitive	1.99 ± 0.04	1.44 ± 0.11
Inhibitor II	223.99 (= 11.20×10^{-8} M)	Non-competitive	1.76 ± 0.10	1.45 ± 0.10

[1] number of inhibitor molecules bound per molecule of enzyme.

[2] denatured bovine hemoglobin.

Table VIII. The effect of temperature on the K_i, type of inhibition, and number of inhibitor molecules bound per molecule of trypsin in 0.05 M sodium phosphate buffer (pH 7.9)

A. Inhibitor I		No. of sites[1]			
Temperature (C)	K_i (μg/2 ml)	0.25 mM^2	0.5 mM^2	3 mM^2	Type of inhibition
30	0.92	1.03 ± 0.02	-	0.95 ± 0.09	Competitive
34	1.36	-	1.16 ± 0.04	1.15 ± 0.12	Competitive
37	1.81	-	1.99 ± 0.04	1.44 ± 0.11	Non-competitive
44.5	2.84	-	1.61 ± 0.14	1.66 ± 0.23	Non-competitive

B. Inhibitor II		No. of sites[1]		
Temperature (C)	K_i (μg/2 ml)	0.5 mM^2	3 mM^2	Type of inhibition
30	60.88	1.17 ± 0.09	1.80 ± 0.13	Competitive
34	180.94	1.43 ± 0.13	1.06 ± 0.05	Competitive
37	223.99	1.76 ± 0.10	1.45 ± 0.10	Non-competitive
44.5	365.26	0.88 ± 0.04	1.02 ± 0.04	Non-competitive

[1] number of substrate molecules bound per molecule of enzyme.

[2] BAPNA was used as the substrate.

Figure 22. The van't Hoff plots for the formation of trypsin-inhibitor I and trypsin-inhibitor II complexes.

A. trypsin-inhibitor I complex, B. trypsin-inhibitor II complex. 3 mM BAPNA was used as substrate. The reaction was carried out in 0.05 M sodium phosphate buffer (pH 7.9) for 20 minutes at 37 C. The K_i values were from the Table VIII.

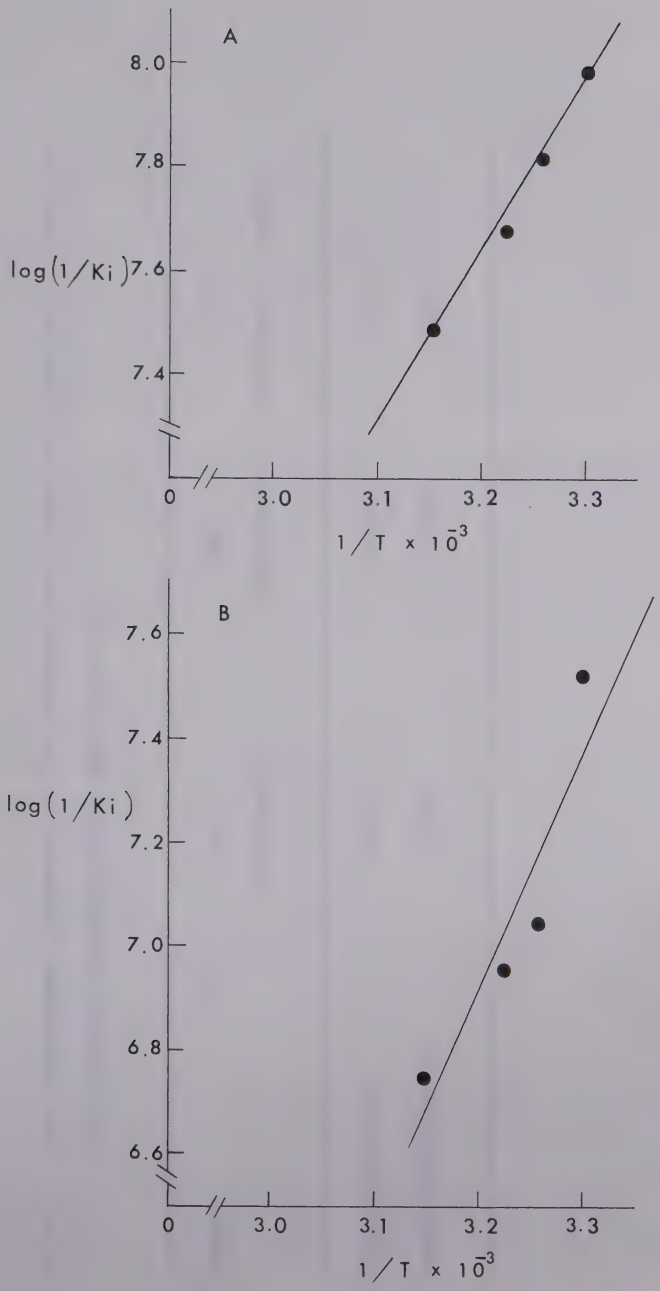

A
8.0
7.8
log(1/Ki)7.6
7.4
0
3.0
3.1
3.2
3.3
1/T x 10⁻³
B
7.6
7.4
log(1/Ki)
7.2
7.0
6.8
6.6
0
3.0
3.1
3.2
3.3
1/T x 10⁻³

Table IX. The thermodynamic parameters of the interaction of bovine inhibitors with trypsin in 0.05 M sodium phosphate buffer (pH 7.9) at 37 C

Complex	ΔF° (Kcal/mole)	ΔH° (Kcal/mole)	ΔS° (Kcal/mole x degree)
Trypsin-inhibitor I	-11.38	-14.80	-11×10^{-3}
Trypsin-inhibitor II	-9.88	-21.89	-38.06×10^{-3}

cannot be reactivated by adding inhibitor II (Table X).

The above studies indicate that most of the proteolytic and esteratic activities of mosquito trypsin are inhibited by inhibitor I, but only the proteolytic activity is inhibited by inhibitor II. A remarkable fact is that the trypsin-inhibitor II complex retains most of the esteratic activity even in the presence of inhibitor I or other trypsin inhibitors. From this aspect, one can imagine the complexity of blood meal digestion within the mosquito midguts.

5. The digestion of the blood meal by adult female mosquitoes

The purpose of this study is to measure the esteratic activity of proteases and to observe the electrophoretic patterns of blood meal proteins in the adult female mosquito midguts during the course of digestion.

After a rat or human blood meal, five fed female mosquito midguts were dissected at each time interval (Fig. 23) and homogenized in 0.5 ml of 0.9% NaCl solution. The solution was centrifuged and the supernatant was used for both trypsin and chymotrypsin activity measurement in the spectrophotometer. Some of the supernatant was electrophoresed on a cellulose acetate membrane under the same conditions as described before except the field strength and duration. The protein content per midgut was determined also.

The relationships between the enzyme activities (trypsin and chymotrypsin), and the protein content in each fed mosquito midgut during 50 hours of blood meal digestion is shown in Figure 23. The maximum trypsin activity was at 35 hours and 40-45 hours after rat and

Table X. Irreversibility of combinations of inhibitors with trypsin*

Inhibitor first added (μg/2 ml)	First incub.	Second compound	Second incub.	Inhibition %
	15 min.	Buffer	15 min.	0
Inhibitor II (108)	"	Inhibitor I	"	53
	"	Soybean trypsin inhibitor	"	63
	"	PMSF	"	62
	"	TLCK	"	99
Inhibitor I (11.8)	"	Buffer	"	95
	"	Inhibitor II	"	97
Soybean trypsin inhibitor (10)	"	Buffer	"	98
	"	Inhibitor II	"	98
PMSF (1230)	"	Buffer	"	97
	"	Inhibitor II	"	99
TLCK (378)	"	Buffer	"	100
	"	Inhibitor II	"	99

*_Aedes aegypti_ trypsin was 2.74 μg/2 ml.

Figure 23. Trypsin activity, chymotrypsin activity and protein content of the midgut at various times after a blood meal.

mosquitoes fed rat blood:

-●- trypsin

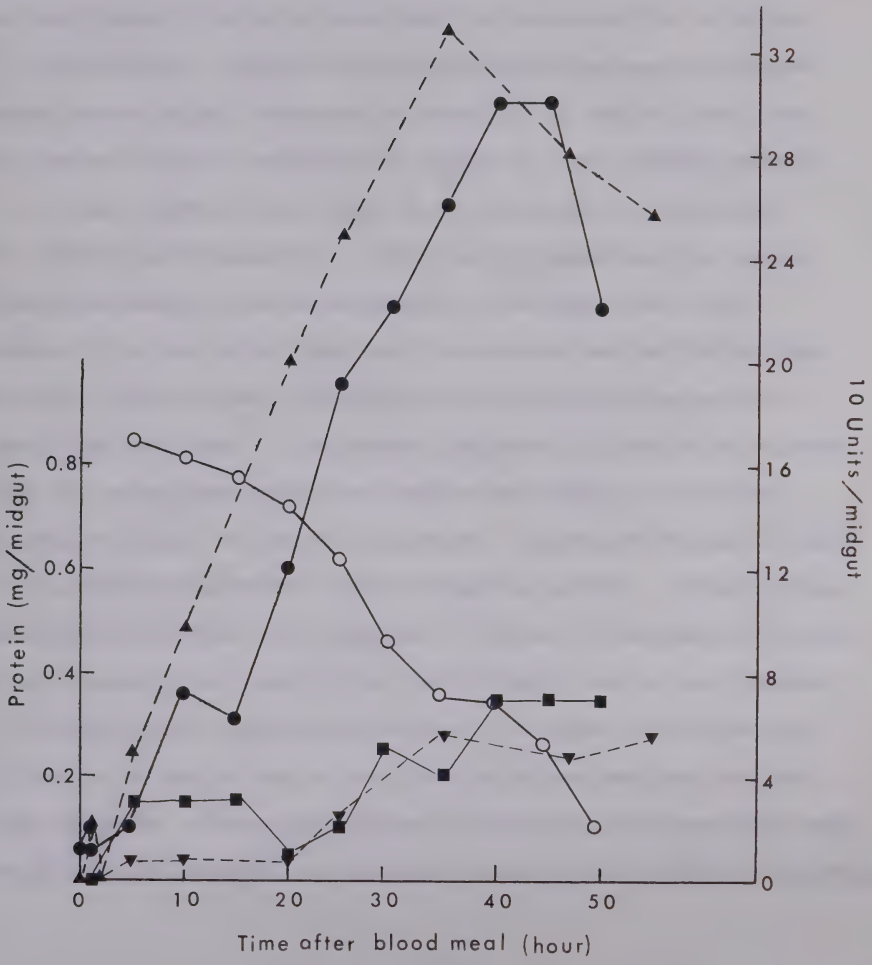
-■- chymotrypsin

-o- protein

mosquitoes fed human blood

-▲- trypsin

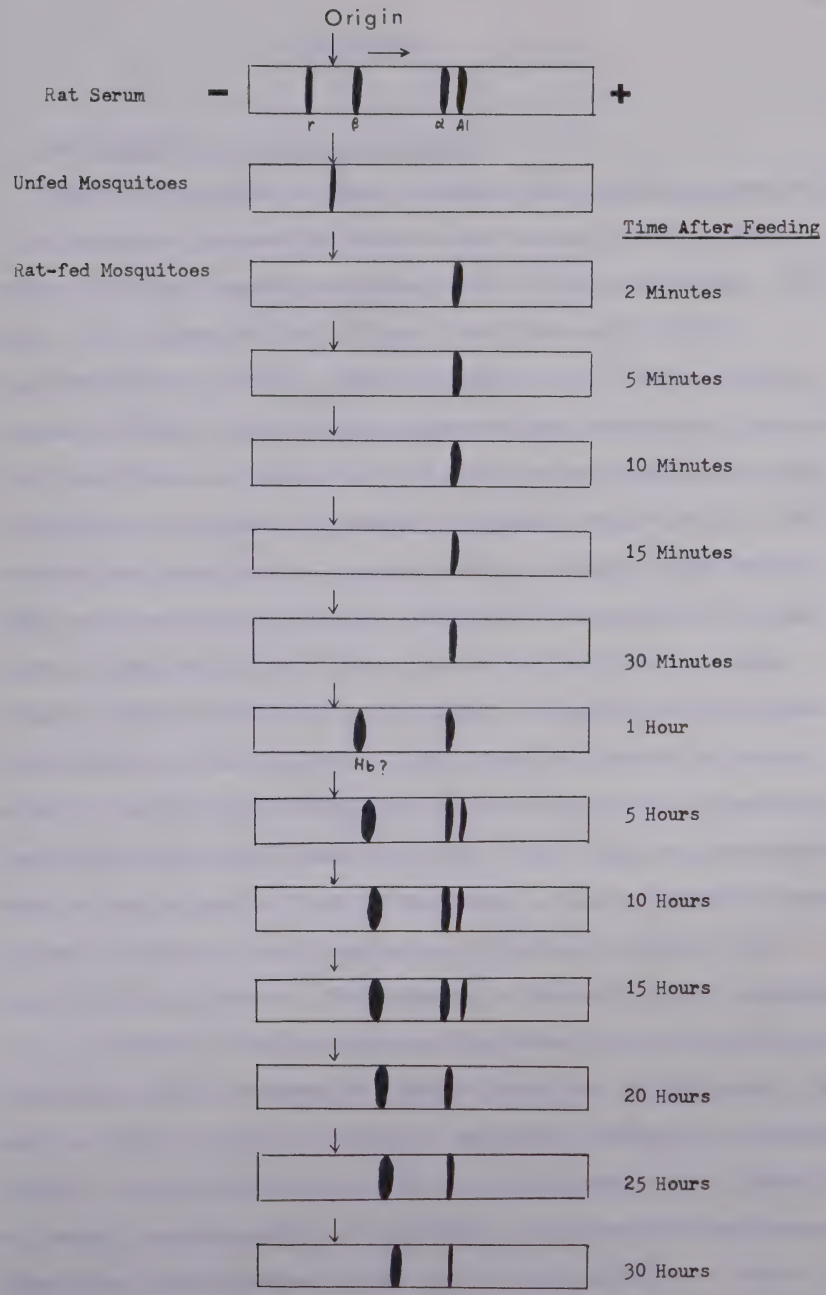
-▼- chymotrypsin


Protein (mg/midgut)
0.8
0.6
0.4
0.2
0
10
20
30
40
50
Time after blood meal (hour)
32
28
24
20
16
12
8
4
0
10 Units/midgut

human blood meal respectively. Very little chymotrypsin was found after either rat or human blood meal. The protein content of mosquito midgut declined gradually during 50 hours after the mosquitoes fed on the rat. The electrophoretic patterns of rat serum, midgut homogenates of unfed mosquitoes and midgut homogenates of mosquitoes at various times after they fed on a rat are represented in figure 24. Four proteins (albumin, α-, β-, and γ-globulin) were found in rat serum, but no protein band was found in unfed mosquitoes. In the rat-fed mosquitoes, one protein band corresponding to the albumin region of rat serum, was found 2 minutes to one hour after digestion. The albumin band was divided into two parts 5-15 hours after digestion and only one band remained 20-25 hours after digestion. It had almost disappeared 30 hours after digestion. Other rat serum proteins were not observed definitely in the midgut homogenates during the course of digestion. This may be because of their low concentration in serum or they are digested quickly. Another protein band appeared one hour after digestion. This may be hemoglobin, since the color of supernatants used for the electrophoresis was not red 2 minutes to 30 minutes after digestion but became red one hour after digestion. If this is so, some of the rat red blood cells have hemolyzed one hour after digestion. The new protein band gradually migrated toward the anode during the electrophoresis and gradually disappeared 30 hours after digestion.

Figure 24. Diagrams of protein separation obtained by electrophoresis of rat serum, midgut homogenates of unfed mosquitoes, and midgut homogenates of rat fed mosquitoes at various times after a blood meal.

The electrophoresis was run in 0.06 M barbital buffer (pH 8.6) 250 volts, 6 ma for 4 hours at 4 C.


Origin
Rat Serum
−
+
γ
β
α
Al
Unfed Mosquitoes
Time After Feeding
Rat-fed Mosquitoes
2 Minutes
5 Minutes
10 Minutes
15 Minutes
30 Minutes
1 Hour
Hb?
5 Hours
10 Hours
15 Hours
20 Hours
25 Hours
30 Hours

DISCUSSION

1. Some aspects of *Aedes aegypti* trypsin

Most of the studies of insect proteases have considered these enzymes to be comparable to mammalian trypsins with respect to the enzymic pH optima (pH 8) and temperature optima (45-50 C) (Lin and Richards, 1956; Evans, 1958; Desnuelle, 1960; Gilmour, 1961; Buck *et al.*, 1962; Wigglesworth, 1965; Gooding, 1966 a; Kafatos *et al.*, 1967 a; Gooding and Huang, 1969). Insect alkaline proteases have been further characterized into trypsin and chymotrypsin by substrate specificity, similarity to inhibitors, and other biochemical techniques. Wagner *et al.*, (1961) reported that whole adult *A. aegypti* contain a trypsin (hydrolysis of BAEE) and a non-trypsin protease (hydrolysis of hemoglobin). Gooding (1966 a) found both trypsin (hydrolysis of BAEE and TAME) and chymotrypsin (hydrolysis of BTEE) in the midgut of blood fed *A. aegypti* and *Culex fatigans*. Yang and Davies (1968) found the presence of tryptic activity (hydrolysis of TAME) in the midgut of 5 species of black-flies. Gooding (in press) also demonstrated that both trypsin and chymotrypsin exist in the midguts of blood fed *Melophagus ovinus* and *Pediculus humanus* but not in those of *Cimex lectularius* or *Rhodnius prolixus*. In the non-blood sucking insects, the proteases of *Tenebrio molitor* (Applebaum *et al.*, 1964) and *Tribolium castaneum* (Applebaum and Konijn, 1966) have activities similar to mammalian trypsin (hydrolysis of polylysine). Rao and Fisk (1965) reported a trypsin in the midgut homogenate of *Nauphoeta cinerea*. Lecadet and Dedonder (1967 a; b) found both trypsin (hydrolysis of benzoyl arginine amide) and chymotrypsin (hydrolysis of carbobenzoxy-phenylalanyl phenylalanine) in the gut of *Pieris brassicae*. Kafatos *et al.*,

(1967 a; b) purified and characterized a trypsin-like cocoonase from *Bombyx mori*. Zwilling (1968) isolated two proteases from *Tenebrio molitor*: β-protease (hydrolyzes BAEE but not N-acetyl-L-tyrosine ethyl ester; is inhibited by all trypsin inhibitors) and α-protease (hydrolzes neither BAEE nor N-acetyl-L-tyrosine ethyl ester; is not inhibited by trypsin inhibitors, except PMSF, soybean and limabean inhibitors). Sonneborn *et al.*, (1969) found a chymotrypsin (hydrolyzes ATEE and N-acetyl-L-phenylalanine β-naphthyl ester; is inhibited by N-tosyl-L-phenylalanyl-chloromethane and PMSF) in the midgut of the larva of the hornet, *Vespa orientalis*. Gooding and Huang (1969) reported trypsin and chymotrypsin in the guts of both male and female *Pterostichus melanarius*, and presented evidence to support the idea that the tryptic and chymotryptic activities are associated with two separated proteins.

The midgut of blood fed *A. aegypti* has much higher tryptic activity (hydrolysis of BAEE and BAPNA) than chymotryptic activity (hydrolysis of BTEE) (Fig. 23). This confirms the report of Gooding (1966a). In the present study, only trypsin is considered and the assay temperature is 37 C instead of 46 or 49 C which is the optimum temperature of *A. aegypti* proteases (Gooding, 1966 a). Assays were done at 37 C to avoid the possibility of denaturing the enzyme and/or the serum inhibitors at the higher temperature (Evans, 1958). The optimum pH of the trypsin hydrolysis of BAPNA was 7.9 at 37 C (Fig. 18) which is the same optimum pH reported for *A. aegypti* proteases hydrolysis of denatured hemoglobin at 49 C (Gooding, 1966 a). In *A. aegypti* extract, only one trypsin peak was found after Sephadex G-100 gel column but two trypsin peaks were found

after DEAE-cellulose chromatography (Figs. 1 and 2). The appearance of two enzymic peaks is not unexpected, since the enzymes may be partially digested during purification. The same problem has arisen during the purification of other enzymes (Wagner *et al.*, 1961; Gazith *et al.*, 1968; Gooding and Huang, 1969) or protein (Wu and Laskowski, 1960). The molecular weight of the trypsin is about 21,500 which is close to 21,800 of *Pterostichus melanarius* (Gooding and Huang, 1969), but it is smaller than 24,500 of cocoonase (Kafatos *et al.*, 1967 a) and 23,800 of mammalian trypsin (Keil, 1965). Both the trypsin and the bovine trypsin are inhibited by the same inhibitor of the bovine serum (Fig. 5). The trypsin is inhibited by the soybean trypsin inhibtor, PMSF, and TLCK (Table X), and the effect of the latter two synthetic inhibitors further indicates that serine and histidine are probably involved in the active center of this enzyme (Farhney and Gold, 1963; Shaw, 1967). Many physical, chemical, and biochemical properties of this enzyme are still unknown, nevertheless, the above evidence (enzyme kinetic properties, substrate specificity, and inhibitors) further support the idea of a common evolutionary origin of trypsin-like proteolytic enzymes (Neurath *et al.*, 1968).

2. *Aedes aegypti* trypsin inhibition capacity of animal sera

All the sera studied, both from poikilothermic animals and homeothermic animals, inhibit *A. aegypti* trypsin (Tables II A and B). The order of inhibition capacity of fresh sera of mammals is: sheep > cow > pig > rat > elk > human (μg trypsin/μl serum) and cow > rat > sheep = pig > human = elk (μg trypsin/μg serum). The lyophilized sera of horse, dog, and rabbit have lower inhibition capacities than other mammals studied. The

mammalian trypsin inhibition value of normal human serum is 0.42 - 1.9 (mg trypsin/ml serum) (Jacobsson, 1955; Bundy and Mehl, 1958; Dyce and Haverback, 1960; Zipf *et al.*, 1961; Schön *et al.*, 1962; Métais *et al.*, 1965, 1966; James *et al.*, 1966 a); of normal rat is 1 - 2 (mg trypsin/ml serum) (Gülzow *et al.*, 1961; Forell and Dobovicnik, 1961); of normal dog is 1 (mg trypsin/ml serum) (Lohmann, 1962); of normal guinea pig is (1.55 mg trypsin/0.05 ml serum) (Ungar, 1945); and of normal rabbit serum is 6.6 - 7.5 (units/ml serum) (Grob, 1943). Launoy (1919) studied numerous species of animals and showed antiproteolytic activity to be a constant property of mammalian and bird sera. Duthie and Lorenz (1949) reported the relative mammalian trypsin inhibition capacity of four mammalian sera to be: sheep > horse > human > rabbit. They also stated that there is a marked species variation between the anti-tryptic power of different animal sera, but little variation between the sera of individual members of the same species. In general, the *A. aegypti* trypsin inhibition capacity of animal serum, the same as anti-mammalian trypsin, is uniform in character but with a variation in degree of potency between species.

The difference of trypsin inhibition capacity among species may come from the difference in amount of inhibitor and the specific inhibition capacity of each inhibitor in serum. These factors are affected mainly by the genetic, physiological, and pathological condition of the animal (Vogel *et al.*, 1968). For the purpose of comparison among species, the serum inhibitors can be placed in one of three groups on the basis of their molecular weights: α_2-macroglobulin inhibitor (≥160,000), inter α-globulin inhibitor (77,600 - 107,000), and α_1-globulin inhibitor

(31,800 - 66,100). All the mammals studied, have the α_2-macroglobulin inhibitors (with the exception of rat) and α_1-globulin inhibitors. Only human serum contains the inter α-globulin inhibitor. Heim (1968) reported that a slow α_2-globulin of the rat which is chemically, immunologically and probably functionally similar to the α_2-macroglobulin of other non-rodent mammals, but this α_2-globulin is absent from the serum of the normal, non-pregnant adult rat. Also an α_1-macroglobulin has been found in rat serum, which is related to the α_2-macroglobulin of other mammals (Boffa *et al.*, 1964; Ganrot, 1968). In general, α_2-macroglobulin is found in the sera of normal, non-pregnant humans, and other mammals, but not in mice and other rodents (Poulik and Smithies, 1958; James, 1965; Picard *et al.*, 1966; Demaille *et al.*, 1966). Both chicken and turkey sera show only one broad inhibition peak with a molecular weight around 57,500 and 50,100 respectively. It is still uncertain whether bird sera contain only one trypsin inhibitor or whether there are several inhibitors with similar molecular weights. However, an α_2-macroglobulin has been found in serum from duck (Finch, 1966), and serum proteins in some birds have an electrophoretic pattern similar to that of human serum proteins (Dzulynska and Krajewska, 1964). Trypsin inhibitors in turtle and frog serum have a distribution similar to the human serum. One teleost serum has two trypsin inhibitors but without α_2-macroglobulin inhibitor. Dogfish serum has one inhibitor only, and *P. americana* has one low molecular weight inhibitor ($\leq$11,500). Nakamura (1966) studied the mammalian trypsin inhibitors of vertebrate sera by using the cross-electrophoretic technique. He reported that bovine, horse, sheep, cat, dog, rabbit, and guinea pig sera all have two inhibitors: α_1-and α_2-globulin inhibitors; but porcine serum has only

an α_1-globulin inhibitor. He also reported that tortoise, frog, carp, and eel have 3, 2, 2, and 2 trypsin inhibitors respectively. In human serum, three mammalian trypsin inhibitors have been characterized: α_1-globulin inhibitor (Jacobsson, 1955; Moll *et al.*, 1958; Bundy and Mehl, 1959; Mansfeld *et al.*, 1959, 1960; Schultze *et al.*, 1955, 1962, 1963; Störiko and Schwick, 1963; Rimon *et al.*, 1966), inter α-globulin inhibitor (Heide *et al.*, 1965; Schwick *et al.*, 1966), and α_2-macroglobulin inhibitor (Haverback *et al.*, 1962; Schultze *et al.*, 1963; Mehl *et al.*, 1964; James *et al.*, 1966 b; Ganrot, a, b, c and d; Ganrot and Scherstén, 1967). Using bovine serum, Peanasky and Laskowski (1953) partially purified a mammalian trypsin inhibitor, Wu and Laskowski (1960) reported an α_1-crystalline mammalian trypsin inhibitor and Gray *et al.* (1960) also reported a mammalian proteolytic inhibitor. Martin (1961, 1962) reported an α_1-mammalian trypsin inhibitor in the sheep serum. Kieken *et al.* (1965) found an α_1- and an α_2-globulin mammalian trypsin inhibitor in dog plasma. In rabbit plasma, McCann and Laskowski (1953) found a mammalian trypsin inhibitor. Picard and Heremans (1963) isolated a rabbit α_2-macroglobulin. Nartikova and Paskhina (1968) also purified and characterized an acid stable mammalian trypsin inhibitor from rabbit serum. The above information provides us with another example of the so called biochemical unity and dissimilarity among organisms. These *A. aegypti* trypsin inhibitors are considered to be similar proteins, since they come from the same tissue (serum) of organisms, have similar molecular weights and similar biological function. However, their evolutionary origin and much other information (physical and chemical properties, biosynthesis, amino acid sequence, etc) are still unknown. In this study, I found a trypsin inhibitor (molecular weight

~11,500) in the haemolymph of *P. americana*. Engelmann (1969) reported that a powerful inhibitor for the proteases of the cockroach, *Leucophaea maderae*, is found in the anterior midgut and the caeca of the same insect species. The difference in the trypsin inhibtion capacity of each animal serum may, theoretically, influence the rate of blood digestion by insects and this in turn may influence host selection or host preferences. It has been reported from field observations that some mosquito species feed selectively on particular hosts. For example, *Culex territans* feed on amphibians and reptiles (Steward and McWade, 1961), and *A. aegypti* feed on several taxa of vertebrates (Downe, 1960; McClelland and Weitz, 1963; Schaefer and Steelman, 1969). However, a few mosquito species feeding on insects have been reported by Downes (1958). Recently, Harris *et al.*, (1969) and Harris and Cooke (1969) reported that caged *A. aegypti* and *Culex tarsalis* are attracted to some insect larvae.

3. Some similarities of trypsin inhibitors in mammalian sera

Inhibitor I is electrophoretically found in association with the α_1-globulin of bovine serum (Fig. 10). The mobility of this inhibitor is -5.80×10^{-5} and -1.30×10^{-5} $cm^2/v/sec$ at pH 8.6 and 7.0 respectively. The molecular weight is estimated as 43,500 by the gel filtration method (Fig. 11). This substance inhibits both mosquito trypsin and bovine trypsin. A similar inhibitor has been reported by Wu and Laskowski (1960), which has a mobility of -6.51×10^{-5} $cm^2/v/sec$ at pH 8.6, a molecular weight of 71,000 (sedimentation-diffusion method) and of 39,000 (activity method), and inhibits mammalian trypsin, α-and B-chymotrypsin, elastase,

and plasmin. Gray *et al.*, (1960) reported an antiplasmin-antitrypsin protein from bovine blood with a mobility of -5.61 x 10^{-5} $cm^2/v/sec$ at pH 8.6 and a molecular weight of 72,000 (sedimentation-diffusion method). Nanninga and Guest (1964) found an antiplasmin inhibitor from bovine plasma with a molecular weight of 57,000 (sedimenation-diffusion method). Of human α_1-trypsin inhibitor, Bundy and Mehl (1959) reported that it has a molecular weight of 45,000 and it inhibits mammalian trypsin and α-chymotrypsin, and Schultze *et al.*, (1962) reported a mobility of -5.42 x 10^{-5} $cm^2/v/sec$ at pH 8.6. Martin (1961, 1962) found that sheep α_1-trypsin inhibitor has a molecular weight of 40,600 (sedimentation-diffusion method) and a mobility of -5.2 x 10^{-5} $cm^2/v/sec$ at pH 8.6, and it inhibits mammalian trypsin, α-chymotrypsin, human plasmin, and rat skin proteinase A, but not thrombin or elastase.

Inhibitor II is associated with the α_2-globulin of bovine serum (Fig. 10), and it has a mobility of -5.21 x 10^{-5} and -1.23 x 10^{-5} $cm^2/v/sec$ at pH 8.6 and 7.0 respectively. The molecular weight of this inhibitor is assumed to be 1,000,000, the same as the foetal calf α_2-macroglobulin (Marr *et al.*, 1962). It also inhibits both the mosquito trypsin and the bovine trypsin. Of human α_2-macroglobulin inhibitor, it has been reported with a molecular weight of 820,000 (Schönenberger *et al.*, 1958) or 845,000 (Schwick *et al.*, 1966), a mobility of -4.2 x 10^{-5} $cm^2/v/sec$ (Schönenberger *et al.*, 1958), and it inhibits mammalian trypsin (Bennich and Goa, 1958; Schön *et al.*, 1962) and plasmin (Norman, 1958, Schultze *et al.*, 1963). The molecular weight of α_2-macroglobulins from rabbit, pig, and rat have been reported as 850,000 (Picard *et al.*, 1964), 960,000 (Jacquot-Armand and Guinand, 1967) and 900,000 - 1,000,000 (Heim, 1968) respectively.

The above comparisons, indicate that inhibitor I and II are similar to the α_1-trypsin inhibitor and α_2-macroglobulin inhibitor respectively of other mammalian sera.

4. Interaction of bovine serum inhibitors and *Aedes aegypti* trypsin

In this study, I found that one µg of inhibitor I inhibits 1.73 µg of *Aedes* trypsin (Fig. 12), but 0.83 µg of bovine trypsin. This means that one molecule of inhibitor I can inhibit 3.5 molecules of *Aedes* trypsin but 1.50 molecules of bovine trypsin. Inhibitor I action on *Aedes* trypsin is about twice as great as on bovine trypsin, and this difference may have arisen from the purity and/or from the source of the enzymes. Wu and Laskowski (1960) reported that one µg of bovine α_1-globulin inhibitor inhibits 0.62 µg of mammalian trypsin at the equilibrated point when TAME substrate was used, and they thought one molecule of inhibitor reacted with one or two molecules of enzyme. I also found that 100% inhibition of either *Aedes* trypsin or bovine trypsin cannot be achieved even with an excess of inhibitor. Wu and Laskowski (1960) found the same result for mammalian trypsin. Schultze *et al.* (1962) reported that one µg of human α_1-globulin inhibitor gives 100% inhibition of 0.4 - 0.6 µg of mammalian trypsin. Martin (1962) found one µg of sheep α_1-globulin inhibitor inhibits the esteratic activity of 0.56 µg of mammalian trypsin and the proteolytic activity of 0.77 µg of mammalian trypsin. Gray *et al.* (1960) found a β-globulin inhibitor in bovine serum with a mammalian trypsin inhibition capacity of about 0.19 (µg trypsin/µg inhibitor) when casein was used as the substrate, and the molar ratio of inhibitor to trypsin was approximately 1:1.

The average value of *Aedes* trypsin inhibition capacity of inhibitor II is 0.04 ± 0.01 (Fig. 14) and the inhibition capacity is only 1/43 of the capacity of inhibitor I. The molar ratio of enzyme to inhibitor II is 1.72 ± 0.45 which is similar to the 2:1 ratio (Ganrot, 1966 b; Jacquot-Armand, 1967), but not the 1:3 ratio (James *et al.*, 1966 b) of mammalian trypsin and human α_2-macroglobulin inhibitor. One μg of human α_2-macroglobulin inhibitor inhibits 0.017 - 0.04 μg of mammalian trypsin (Vogel *et al.*, 1968). The above information indicates that inhibitor I is the main trypsin inhibitor (*Aedes* trypsin or mammalian trypsin) in most vertebrate sera (Tables II A and B).

Both inhibitors combine spontaneously, stoichiometrically, and irreversibly with *Aedes* trypsin. The complex of trypsin and inhibitor I was isolated from a Sephadex gel column and it had neither the trypsin esteratic activity nor inhibition capacity (Fig. 15 D). The complex of trypsin and inhibitor II, on the other hand, retained some esteratic activity after Sephadex gel filtration and electrophoresis (Figs. 15 E and 16). The pH effect on the esteratic activity of this complex is similar to the effect on free enzyme (Fig. 18), and the complex has a K_m about one half of the free enzyme (Table V; Fig. 17). However, the number of substrate molecules (BAPNA) bound per molecule of enzyme is about one for both free enzyme and complex (Table V). Troyer and Moskowitz (1968) found the mammalian trypsin-human α_2-macroglobulin complex retains only 5% of proteolytic activity (hydrolysis of casein) but 80% of hydrolytic activity against BAPNA. According to Ganrot (1966 a), the inability to cleave proteins is due to steric hindrance of the complex. Howard (1966) reported that mammalian trypsin-human

α_2-macroglobulin complex is about 65% as active as free trypsin using the substrate BAPNA. The complex is irreversible and has a higher K_m and lower K_{cat} than those of free enzyme for both TAME and BAEE. She also found the substrate activation with this complex. Recently, Boyde and Pryme (1968) found that human α_2-macroglobulin binds the trypsin, chymotrypsin, papain and cationic aspartate aminotransferase. Boyde (1969) stated that all the proteins known to be susceptible to binding by α_2-macroglobulin are cationic, and this leads to the suggestion that binding is the result of electrostatic interaction rather than any specific effects. Ganrot (1966 c) was able to separate the plasmin inhibitor from the trypsin protector of human α_2-macroglobulin by gel filtration on Sephadex G-200, so he indicated that these are two different α_2-macroglobulins and not two active centers of the same protein. However, in the present study, only one bovine α_2-macroglobulin was found to have the trypsin inhibiting and trypsin protecting properties.

The esteratic activity of trypsin-inhibitor II complex is only partially inhibited by excess amount of inhibitor I, soybean trypsin inhibitor, or PMSF which can completely inhibit the free enzyme (Table X). However, this complex is completely inhibited by TLCK. James *et al.* (1967) found that the human α_2-macroglobulin protects the mammalian trypsin from DFP-inactivation. The protection of trypsin from PMSF but not TLCK inhibition by inhibitor II is rather interesting. One might assume that inhibitor II is capable of binding PMSF (but not TLCK) irreversibly, so that the serine active site on the enzyme molecule is still accessible to the substrate BAPNA. The protection of trypsin from other protein inhibitors by inhibitor II is rather complicated, and it

will be considered later.

5. Mechanism of inhibition

It is generally agreed that the inhibition of proteolytic enzymes by protein inhibitors requires the formation of a complex which is strongly associated. But there is no general agreement as to what the driving force is for this interaction (Feeney and Allison, 1969). The following two theories have been suggested for the mechanism of inhibition.
(1). Laskowski and his co-workers proposed that the trypsin inhibiting reaction consist of a cleavage of one especially sensitive bond in the inhibitor by trypsin, and of subsequent formation of a covalent bond between trypsin and inhibitor (probably an ester bond between the active site of trypsin and the newly formed COOH-terminal of the inhibitor) (Finkenstadt and Laskowski, 1965, 1967; Ozawa and Laskowski, 1966).
(2). Feeney and his co-workers proposed that a particular lysine or arginine residue in the trypsin inhibitor and a tyrosine, tryptophane, alanine, or methionine residue in the chymotrypsin inhibitor serves as the recognition site or binding site of the inhibitor to a binding site of the enzyme. In addition, other noncovalent bonds or forces strengthen the association, possibly as a result of a conformational change causing a better fitting. The peptide of this particular residue is relatively resistant to proteolysis and is cleaved very slowly or incompletely, if at all, by the enzyme (Haynes and Feeney, 1968).

The affinity of trypsin for BAPNA, (that is $1/K_m$), is about five fold greater than that for denatured bovine hemoglobin. The affinity of this enzyme for inhibitor I and for inhibitor II, (that is $1/K_i$), is

increased about 2.5 and 6 fold respectively when the substrate is changed from denatured bovine hemoglobin to BAPNA. The affinity of trypsin for its two bovine inhibitors is about 2-4 x 10^4 times greater than for its two substrates (Tables V and VII). In addition, inhibitor I has a greater affinity for trypsin than inhibitor II. In contrast to these results, kinetic studies show that mammalian trypsin added to human serum has a greater affinity for α_2-macroglobulin than for other trypsin inhibitors (Ganrot, 1966 a; Ganrot and Laurell, 1966). Based on the method of Green and Work (1953), Wu and Laskowski (1960) reported that the K_i of mammalian trypsin-bovine α_1-globulin was 2.04 x 10^{-10} M when TAME was used, and Martin (1962) reported that the K_i of mammalian trypsin-sheep α_1-globulin complex was 4-6 x 10^{-9} M when either TAME or casein was used as the substrate. Based on the Hill plot (Fig. 21; Tables V and VII) (Loftfield and Eigner, 1969), one substrate molecule (either BAPNA or denatured bovine hemoglobin) reacts with one enzymic site, and about two inhibitor molecules (both bovine inhibitors) inactivate one enzymic site. The latter result is not in agreement with the molar ratio of enzyme-inhibitor complex mentioned before.

The bovine serum and its two purified inhibitors are found to non-competitively inhibit the action of trypsin on BAPNA. Bieth *et al.* (1968) reported that the mammalian trypsin inhibition by human serum is also non-competitive. When denatured bovine hemoglobin was used as the substrate, the type of inhibition by these two bovine inhibitors is competitive, and this again is in agreement with the study of beetle proteases (Gooding and Huang, 1969). From the observation of mammalian trypsin-soybean trypsin inhibitor complex formation, Green (1953) found

that the affinity of trypsin for the inhibitor is much greater than that for natural substrates and thus no competitive effect should be expected, but with synthetic substrates which show a much greater affinity for trypsin, so the inhibition should be considered competitive. This idea, however, cannot be applied to distinguish the type of inhibition found in the present study, since there is no significant difference between the dissociation constants of the enzyme-substrate complex and the enzyme-inhibitor complex. Howard (1966) noted that the degree of apparent inhibition of mammalian trypsin by excess of human α_2-macroglobulin varied from over 80% with 1% casein as the substrate to less than 30% with high concentrations of TAME, and inhibition was considered to be a mixed type which altered both K_m and K_{cat}. From the plot of S/V vs S (and 1/V vs 1/S), I found that both the K_m and the V_{max} of trypsin action on BAPNA at 37 C are changed with the concentration of both inhibitors. Therefore the type of inhibition of these two inhibitors is also a mixed type and not a purely non-competitive inhibition (Table VI). At this moment, the only explanation that can be given for the change in type of inhibition of these two bovine inhibitors when different kinds of substrates are used is that the denatured bovine hemoglobin has a closer structure (protein molecule) than the BAPNA substrate to the inhibitors. The trypsin-inhibitor II complex has been shown to be enzymically active against low-molecular weight esters or amides (BAEE, BAPNA) but not against proteins (denatured bovine hemoglobin). The enzymic activity of this complex can be inhibited by TLCK but not by PMSF, soybean trypsin inhibitor, or the inhibitor I (Table X). The enzymic activity of mammalian trypsin-human α_2-macroglobulin complex was reported also to be inhibited

by benzamidin or Kunitz's inhibitor (Michalski *et al.*, 1966) but not by the DFP, the soybean trypsin inhibitor, or the serum α_1-inhibitor (Haverback *et al.*, 1962; Mehl *et al.*, 1964; Ganrot, 1966 d; James *et al.*, 1966 a, b, 1967; Belitser *et al.*, 1967). Since the *Aedes* trypsin possesses a lower affinity for inhibitor II than for inhibitor I, the mechanism of the protecting effect of inhibitor II is something other than the assumption made by Bieth *et al.* (1968).

In general, the rate limiting step between the interaction of serine proteases and their substrates is the acylation and/or the deacylation (Zerner and Bender, 1963; Bender and Kaiser, 1962). The rate limiting step between the interaction of serine proteases (trypsin and chymotrypsin) and protein inhibitors most likely involves a conformational change which results in a better fitting of an inhibitor recognition site to a binding site of the enzyme by other non-covalent bonds or forces (Haynes and Feeney, 1968). In addition, Parker and Lumry (1963) and Sturtevant (1962) studied the question of the effect of substrate or inhibitor binding upon the conformation of the chymotrypsin. Wilson (1967) also considered the conformational change of acetylcholinesterase by binding of the ammonium ion at the anionic site of enzyme.

6. Effect of temperature on the reaction of enzyme with substrate and inhibitors

The enzyme-catalyzed reactions, unlike most chemical reactions, are affected by temperature in two different ways so that the velocity of enzymic reaction increases with temperature up to an optimum temperature,

above which the rate decreases rapidly. This optimum results from the combination of two processes with increasing temperature. Below the optimum temperature, the main effect is on the catalyzed reaction and above the optimum, thermal inactivation of the enzyme becomes the predominant factor (Tammann, 1895). It is also known that significant inactivation of the enzyme may occur at temperatures below the optimum. Therefore, the selected temperature in this study is 37 C in most assays, although *A. aegypti* trypsin has an optimum temperature at 46-49 C.

The activation energy (E) of the trypsin catalyzed reaction was obtained by determining the enzymic velocity at four temperatures (30, 34, 37 and 44.5 C) which are all below the optimum temperature of this enzyme, and plotting log velocity against the reciprocal of the absolute temperature (T). The Arrhenius equation indicates that a straight line with a slope equal to -E/2.303 x R (R is the gas constant) can be obtained. Therefore, if the slope is known the activation energy can be calculated, since E = -(slope x 4.566). The heat of activation (enthalpy or ΔH°) for the formation of enzyme-inhibitor complex can be obtained by the van't Hoff plot in which the equilibrium constant, K (= $1/K_1$) is applied (Fig. 22).

The activation energy of the trypsin is 12,370 cal/mole, and the corresponding value for the heat of activation (ΔH° = E - RT) is 11,650 cal/mole at 37 C (Dixon and Webb, 1964). This activation energy value falls within the range of values that has been obtained for trypsin and chymotrypsin (Butler, 1941; Sizer and Josephson, 1942) and the protease of larval blowfly (Evans, 1958). The activation energy of the enzyme is a constant regardless of the substrate used but it may differ between

enzymes (Dawes, 1964). This energy, from a thermodynamic viewpoint, can be regarded as the energy required to place the reacting molecules in an active state. If E is large the rate of reaction increases rapidly in relation to an increased temperature.

The affinity of trypsin for two bovine inhibitors decreases with increased temperature (Table VIII). Both inhibitors competitively inhibited the hydrolysis of the BAPNA at 30 and 34 C but they are non-competitive inhibitors at 37 and 44.5 C. Furthermore, for both inhibitors, changes in temperature result in changes in the numbers of inhibitor molecules inactivating one enzyme molecule. These results again indicate that the temperature affects primarily the activities of the ionized molecules at the active or binding sites of enzyme, substrate, and inhibitor. The heat of activation of trypsin-inhibitor II complex is -21.89 Kcal/mole which is greater than that of trypsin-inhibitor I complex (-14.8 Kcal/mole). These two values are much greater than that reported for trypsin-soybean trypsin inhibitor (Dobry and Sturtevant, 1952; Steiner, 1954). The large negative ΔH° of the two complexes studied could be due to the formation of a covalent bond between the enzyme and the inhibitor or the formation of non-covalent bonds or forces which are the result of conformational changes of the complex after the association of inhibitor with enzyme (Feeney and Allison, 1969). Another possibility is due to the interaction of the complex with the phosphate buffer which has a relative high heat of ionization.

Since temperature affects ionization it will also affect the activities of the ionized molecules of enzyme, substrate, and inhibitor

and consequently it affects the affinity of the enzyme for substrate and inhibitor ($1/K_m$ and $1/K_i$) and the maximal velocity of reaction (V_{max}). This means that the measurement of the velocity of reaction at different temperatures, but at the same pH, does not give correct results for the activation energy or enthalpy, because the activities or the concentrations of the reactive ionic species have been altered by the change in temperature. Dawes (1964) stated that an accurate value for the activation energy can only be obtained if the heats of dissociation of all the ionizing processes leading to the formation of the active complex are subtracted from the overall observed value.

The free energy of association (ΔF°) can be evaluated from the equilibrium constant, K (= $1/K_i$ or $1/K_m$), by the equation $\Delta F^\circ = -2.303 \times R \times T \times \log K$ where R is the gas constant and T the absolute temperature. The association constants and free energies for trypsin complexes with the bovine inhibitors of this study are relatively small compared to those for mammalian trypsin complexes with various trypsin inhibitors (Laskowski and Laskowski, 1954). The entropy (ΔS°) of association can be obtained directly from the free energy and enthalpy of association data, since

$$\Delta F^\circ = \Delta H^\circ - T\Delta S^\circ$$

Both comlexes have a very small negative entropy changed, and they are different from the large negative values expected.

7. Digestion of blood meal

In the present study (Fig. 24), the pattern of rat blood meal digestion in *Aedes aegypti* seems to be similar to that reported by Williams (1956)

and Zaman and Chellappan (1967). The serum globulins are digested first and the serum albumin is digested last. All the serum proteins are completely digested some time after 50 hours. The red blood cells may be hemolyzed at one hour after feeding. The hemolysis of blood cells may have arisen from the combination of mosquito digestion and mechanical force of homogenizer. The hemoglobin seems to be degraded gradually during the digestion (West and Eligh, 1952; O'Gower, 1956). Wigglesworth (1943) obtained similar results when investigating the digestion of blood by *Rhodnius prolixus*. *A. aegypti* mosquito has high tryptic activity and a very low chymotryptic activity in the midgut during digestion (Fig. 23). The time after the blood meal required to reach the maximum tryptic activity is around 35 hours for rat blood meal and 40-45 hours for human blood meal, which is considerably longer than that found by Fisk and Shambaugh (1952) and Gooding (1966 b). These differences could be due to the difference in source of the blood meal, mosquito strain, experimental condition, or assay method.

The maximum volume of human blood taken by the strain of *A. aegypti* used here is 3.27 μl (Gooding, personal communication), this is approximately to 1.31 μl plasma. The *A. aegypti* trypsin inhibition capacity of human serum is 1.62 μg/μl (Table II A). Therefore, the amount of *A. aegypti* trypsin inhibited by a normal human blood meal is 2.12 μg. However, the maximum amount of trypsin secreted by a mosquito after a human blood meal is 1.70 μg (Fig. 23). From the above comparison, one may suggest that mosquitoes must have some adaptive mechanisms which offer facilities for digesting the inhibitors and/or mosquitoes may secrete excess amount of enzyme in order to digest the blood meal

completely. In fact, the blood meal digestion in *A. aegypti* midgut proceeds inward from the periphery and the detailed processes of digestion have been reported by Stohler (1957). The relatively slow digestion of blood meal by mosquitoes is probably associated with two reasons: first, the natural blood proteins are quite resistant to hydrolysis by mosquito proteases and secondly, the inhibition effect of serum inhibitors on the proteolytic activity of enzymes. This type of inhibition is most likely to be a competitive one. In order to digest the blood proteins, they must be denatured first by some unknown physical or chemical treatment in the mosquito body, or simply the mosquito proteases themselves can produce denaturation of the proteins by virtue of their ability to form complexes (Green and Neurath, 1954; Linderstrøm-Lang *et al.*, 1938).

It has been reported that in a number of blood sucking Diptera, such as *A. aegypti* (Fisk, 1950; Fisk and Shambaugh, 1952; Shambaugh, 1954; Gooding, 1966 b), *Stomoxys calcitrans* (Champlain and Fisk, 1956), *Culex fatigans* (Gooding, 1966 b), *Glossina morsitans* (Langley, 1966), and several Simuliidae (Yang and Davies, 1968), only a blood meal (presumably a certain fraction of blood protein) stimulates protease activity, but sucrose solutions do not. Engelmann (1969) found that certain proteins in food have the specific capacity to stimulate the synthesis of proteases in the cockroach, *Leucophaea maderae*. In general, the protease synthesis of most insects is a secretogogue control by certain proteins in food which have an unknown molecular specificity.

The histochemical observations on the midgut epithelial cells of *A. aegypti* mosquito strongly indicated that the proteases are secreted into the midgut lumen shortly after taking the blood meal (Bertram and

Bird, 1961; Gander, 1968). However, the present studies and other results (Fisk and Shambaugh, 1952; Gooding, 1966 b), show that the protease activity reaches a maximum at 18 to 40 hours after taking the blood meal. The delay of protease activity apparently arises from the binding and inactivating effects of serum inhibitors on the enzymes. If the serum inhibitors have this enzyme regulating function, the interaction of the inhibitors with the enzymes must be a reversible process *in vivo*. In fact, an equilibrium does exist between the enzyme-inhibitor complexes and the free components, although the dissociation constants are very small in the physiological pH range. It is possible that the reactivation of the proteases could be established by some selective mechanisms of the peritrophic membrane which surrounds the blood meal, or, the enzymes themselves are able to digest the inhibitors slowly and become free enzymes again.

I believe that further work on the artifical feeding of serum protease inhibitors to adult female mosquitoes and subsequently analyzing the process of digestion using immunological techniques will provide some information about how this insect can get rid of its protease inhibitors. Also, further work on the effect of serum protease inhibitors on the protease activities of other blood-sucking insects may give us some clue to the evolution of the host relationships of these blood-sucking insects.

SUMMARY

1. *A. aegypti* trypsin has been purified from the midguts of blood fed adult females. The purification procedure includes ammonium sulphate precipitation, Sephadex gel filtration, and DEAE-cellulose chromatography.

2. This trypsin is inhibited by all animal sera tested; these include nine mammals, two birds, one reptile, one amphibian, three teleosts, one elasmobranch , and one insect.

3. The capacity of various animal sera to inhibit *A. aegypti* trypsin varies qualitatively and quantitatively. Bird sera have a higher inhibiting capacity than other animals studied. Most sera contain two inhibitors although as many as three and as few as one have been found in some sera.

4. Two bovine serum inhibitors have been purified and their inhibition properties characterized. Inhibitor I has been purified by the method of Wu and Laskowski (1960) which included ammonium sulphate precipitation, DEAE-cellulose chromatography and CM-cellulose chromatography. Inhibitor II has been purified by modifying the method of Ganrot and Scherstén (1967) which included dextran sulphate precipitation, ammonium sulphate precipitation, Sephadex gel filtration, and DEAE-cellulose chromatography.

5. The molecular weight of the purified trypsin and inhibitor I is 21,500 and 43,500 respectively. The molecular weight of inhibitor II is greater than 160,000 and is assumed to be the same as the foetal calf α_2-macroglobulin (1,000,000).

6. The molar ratio of the complex of trypsin and inhibitor I is 3.5 ± 0.49 and of trypsin and inhibitor II is 1.72 ± 0.45. The capacity of inhibitor I and inhibitor II to inhibit trypsin is 1 μg : 1.73 ± 0.24 μg and 1 μg : 0.04 ± 0.01 μg respectively.
7. The Hill plot indicates that one substrate molecule (BAPNA or denatured bovine hemoglobin) reacts with one enzymic site, whereas two inhibitor molecules (inhibitor I or II) inactivates one enzymic site of *Aedes aegypti* trypsin.
8. The type of inhibition of trypsin is competitive for both inhibitor I and II when denatured bovine hemoglobin is used, and non-competitive when BAPNA is used as the substrate at 37 C. The type of inhibition of trypsin is non-competitive also for whole bovine serum when BAPNA is used as the substrate at 37 C.
9. The dissociation constant (K_i) of trypsin-inhibitor I complex is 2.08×10^{-8} M and 5.37×10^{-8} M when BAPNA and denatured bovine hemoglobin respectively are used as the substrates at 37 C. The dissociation constant of trypsin-inhibitor II complex is 11.2×10^{-8} M and 61.7×10^{-8} M when BAPNA and denatured bovine hemoglobin respectively are used as the substrates at 37 C.
10. The thermodynamic parameters of the interaction of trypsin with the two bovine inhibitors and BAPNA have been studied. Both enzyme-inhibitor complexes have a relative low free energy ($\Delta F°$), a very large negative enthalpy ($\Delta H°$), and a very small negative entropy ($\Delta S°$) at 37 C. The enzyme has a high positive activation energy.

11. The complex of trypsin and inhibitor II retains partial esteratic activity and a very low proteolytic activity. The residual activity is not inhibited by inhibitor I, soybean trypsin inhibitor, and PMSF, but it has a pH optimum similar to that of free trypsin.
12. The maximum trypsin activity in the midgut of *A. aegypti* appears around 35 hours after a rat blood meal and 40-45 hours after a human blood meal. The rat blood serum globulins disappear from the midgut shortly after the blood meal and the amount of albumin has started to decline by 30 hours after blood feeding.

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